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DIFFUSION OF YEAST INVERTASE THROUGH COLLODION MEMBRANES.*

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The authors have had occasion to dialyze some crude yeast invertase preparations in solutions of different hydrogen ion concentration, and have found that a considerable amount of enzyme (13 to 14 per cent) passes through the walls of a collodion bag when the solutions inside and outside the bag are of low acidity (pH = 6.7). When the dialysis was conducted in a more acid solution (pH = 4.6) only a trace of the enzyme came through the bag, although a considerable loss in activity of the enzyme inside the bag was noticed.

Regarding the significance of the invertase dialyzing through collodion at low hydrogen ion concentrations, it is felt that as yet, it is too early to make any definite statement. However, it may be of interest to call attention to the suggestion made by Michaelis and Davidsohn (1). In their paper they ascribed the activity of invertase to the unionized part of the enzyme, basing their claim on the similarity of the pH activity curve to the logarithm form of an ordinary simple dissociation residue curve, as for example the titration curve of a weak acid. The pH at which Michaelis and Davidsohn claimed the ionization of the invertase to be at a minimum is the pH at which, according to the present work, a minimum quantity of enzyme comes through the bag, while the pH at which they claimed considerable ionization occurs, corresponds to the pH where considerable enzyme comes through the membrane.

HISTORICAL.

Svanberg (2) dialyzed solutions of yeast invertase, contained in collodion bags, and noticed a decrease in the enzyme content of the bag. At

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first he was inclined to the belief that some of the invertase must have passed through the collodion membrane but later (3) on repeating his experiments he found very little enzyme present in the liquid outside the dialysis bag and concluded that loss of enzyme in the bag was due chiefly to destruction. Willstätter and some of his coworkers (4) also mention that they noticed considerable loss of invertase during dialysis and were of the opinion that the loss was primarily due to inactivation of the enzyme.

It may be mentioned also in this connection that invertase solutions have been subjected to ultrafiltration. Holderer (5) filtered crude invertase solutions through a Chamberland filter and found that only in the case of the more alkaline solutions did the enzyme pass into the filtrate. Svanberg (2, 3) who has repeated Holderer's experiments, disagrees, however, with the latter's conclusions, and claims that even acid solutions, pH = 3.8, permit as high as 84 per cent of the invertase to pass through the filter.

EXPERIMENTAL.

Description of Experimental Procedure.

25 cc. portions of yeast invertase preparations, whose pH had been adjusted to the desired values by means of citrate buffers, were placed in collodion bags, and the latter then suspended in 400 to 450 cc. of water containing the same molar concentration of buffer and hydrogen ion as the liquids in the bags. After the dialysis had been in progress for 40 hours or so, samples were withdrawn from the liquids inside and outside of the dialyzing bags and the relative amounts of invertase determined.

Preparation of Collodion Bags.—8 to 10 gm. of collodion (Merck U.S.P. 245 grains of ether per fluid ounce and 24 per cent absolute alcohol) were placed in a glass tube, 15 cm. long and 3.5 cm. in diameter.[1] The tube was rotated rapidly for 2 to 3 minutes and drained for 6 minutes, protected from air currents until the membrane was dry to touch, so as to obtain membranes of as uniform thickness as possible. After draining, the membranes were filled with distilled water and allowed to stand for about 9 minutes, then emptied and placed in 95 per cent alcohol, and permitted to remain in the latter for 24 hours. After the alcohol treatment they were placed in a large beaker of water and allowed to remain there for another 24 hours.

[1] One can of the collodion out of the six used in making the bags gave membranes which proved to be impermeable to the invertase even in the more alkaline range.

Preparation of Enzyme Solutions.—Yeast, from Ruppert's Brewery, was treated with toluene and allowed to autolyze for about a week. To the filtered autolysate an equal volume of alcohol was added, and the precipitate containing the enzyme filtered off. This precipitate was then extracted with distilled water equal in volume to that of the original autolysate. This clear water extract containing the invertase was labelled "Invertase Solution AR." 100 cc. portions of Solution AR were adjusted to various pH values by adding 25 cc. of 0.1 M citrate buffers. 25 cc. portions of these buffered invertase solutions were placed in each of the dialyzing bags and dialyzed against dis-

TABLE I.

Dialysis of Invertase AR Solutions.

Temperature, 18–21°; volume of enzyme solution in each bag before dialysis, 25 cc.

Bag No.	pH inside and outside of bags.	Cc. of liquid inside and outside of bags.				
		Inside after 46 hrs.	Outside before dialysis.	Outside after 46 hrs.	Inside after 86 hrs.*	Outside after 86 hrs.†
(1)	(2)	(3)	(4)	(5)	(6)	(7)
1 a	6.7	40	460	440	41	413
2 a	6.7	45	445	420	46	390
3 a	6.7	45	445	420	46	390
1 b	4.6	40	430	415	40	388
2 b	4.6	45	445	425	47	396
3 b	4.6	45	460	440	47	410

* Corrected for 5 cc. samples removed after 46 hours dialysis.

† Corrected for 20 cc. samples removed after 46 hours dialysis.

tilled water containing the same concentration of buffer and hydrogen ion as the enzyme solution in the respective bags. The results are given in Table I.

Determination of Velocities of Hydrolysis.

5 cc. samples were withdrawn from the inside of Bags 2a, 3a, 2b, and 3b after 46 and 86 hours of dialysis (Table II). These portions were diluted to 400 cc. with water containing 0.01 M citrate buffer (pH = 4.7). The 5 cc. samples from Bags 1 a and 1 b were diluted to 450 cc. 10 cc. portions of the diluted solu-

tions were added to sucrose solutions, containing sufficient sucrose and buffer to yield solutions of 60 cc. of 16 per cent sucrose and 0.01 M citrate (pH = 4.6). The hydrolyses were run at 25° and the changes in rotation measured in 200 mm. tubes with mercury light. The initial rotation of the hydrolyzing sucrose solutions

TABLE II.

Change in Rotation for 30 Minutes of Hydrolysis of Sucrose Solutions by Portions of Enzyme Solutions from Inside or Outside of Bags and the Corresponding Velocity Constants.

A. From Inside of Bags.

Bag No.	Liquids in Column 3, Table I.	$k \times 10^{-5}$	Liquids in Column 6, Table I.	$k \times 10^{-6}$
	degrees		*degrees*	
1 a	1.02	1074	0.86	898
2 a	0.975	1023	0.815	855
3 a	0.95	997	0.795	832
1 b	0.825	863	0.59	606
2 b	0.70	729	0.50	522
3 b	0.75	782	0.545	564

B. From Outside of Bags.

Bag No.	Liquids in Column 5, Table I.	$k \times 10^{-6}$	Liquids in Column 7, Table I.	$k \times 10^{-6}$
	degrees		*degrees*	
1 a	0.34	353	0.54	560
2 a	0.61	629	0.86	897
3 a	0.91	951	1.34	1404
1 b	0.00+*		0.00+*	
2 b	0.00+*		0.00+*	
3 b	0.00+*		0.00+*	

* Change in rotation, 0.3 to 0.7° in 24 hours of hydrolysis, showing that slight amount of invertase came through at pH = 4.6.

was $R_0 = +24.97°$ and the final, $R_\infty = -7.36°$. The hydrolyses were stopped by the addition of a drop of concentrated sodium hydroxide solution.

In the case of the invertase solution outside of the bags, 10 cc. of the liquid were added to 50 cc. of sucrose-buffer solution so that the final volume, 60 cc., contained 16 per cent sucrose, 0.01 M buffer, pH = 4.6.

Determination of Units of Invertase Contained in Solutions Inside and Outside the Dialysis Bags.

By unit of invertase (Table III), is meant the amount of enzyme required to hydrolyze 25 cc. of a 16 per cent sucrose solution to 0° rotation in 1 minute at 25° and pH = 4.7. Units were calculated by first calculating monomolecular velocity K_s (Table II), then substituting these values in the monomolecular formula, and finding the number of minutes necessary for the hydrolysis to reach 0° rotation. The number of minutes thus calculated is

TABLE III.

A. Units of Invertase Contained in Liquids Inside of Bags.

Bag No.	After 46 hrs.	Per cent loss.	After 86 hrs.	Loss in last 40 hrs.
1 a	0.627	4.9	0.537	
2 a	0.598	9.3	0.511	
3 a	0.582	11.7	0.497	
1 b	0.504	23.8	0.354	19.7*
2 b	0.416	33.7	0.318	14.0
3 b	0.457	28.0	0.344	16.0

B. Units of Invertase Contained in Liquid Outside of Bags.

Bag No.	After 46 hrs.	Per cent diffused.	After 86 hrs.	Per cent diffused.
1 a	0.0252	3.8	0.0455	6.9
2 a	0.0429	6.5	0.0568	8.6
3 a	0.0648	9.8	0.0888	13.5
1 b				
2 b				
3 b				

* Corrected for samples removed after 46 hours.

equal to the reciprocal of the number of units of enzyme contained in that number of cc. of enzyme solution which was present in 25 cc. of the 16 per cent sucrose solution undergoing hydrolysis. The units contained in 25 cc. of enzyme solution at the beginning of dialysis were 0.659 in all cases.

SUMMARY.

Crude yeast invertase solutions have been subjected to dialysis in collodion bags, at different hydrogen ion concentrations. The

results obtained show that more invertase passes through the collodion membrane at pH = 6.7 than at pH = 4.6, while the loss of enzyme during dialysis is greater at pH = 4.6 than at pH = 6.7.

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REDUCING EQUIVALENTS FOR SOME RARE SUGARS AS DETERMINED BY COLORIMETRIC METHODS.

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During the course of different research problems in this laboratory some of the rarer sugars have been used. The amounts of these sugars, during different steps, were determined colorimetrically. The reducing equivalents for these sugars could not be found in the literature. It was, therefore, necessary to determine them when glucose was used as a standard. It was thought desirable to extend the determinations by the use of all the more commonly employed colorimetric methods.

The samples of sugars used in this investigation were of the highest purity and were obtained from the Special Chemicals Company. In the case of the more common sugars, and also *d*-arabinose, cellobiose, fucose, and rhamnose, samples from other sources were also used as checks. The purity of the samples was confirmed by means of the polariscope.

The different colorimetric methods used were the Lewis-Benedict method (1) as modified by Willaman and Davison (2), Folin-Wu (3), Benedict copper method, first modification (4), Folin (5), Benedict copper method, second modification (6), Folin, new (7), Folin-Wu, new (7), Sumner (8), and Kingsbury (9).

Several comparisons of reducing equivalents have been previously made for a number of the more common sugars by various methods. No comparison, however, of results with all of the different colorimetric methods has been reported even for the more common sugars. The equivalents were first redetermined for the common sugars by the older methods. Table I includes these results together with the results obtained by various investigators. The other authors listed in Table I are: Willaman and Davison

(2), Thomas and Dutcher (10), Greenwald, Samet, and Gross (11), Rowe and Wiener (12), Pucher and Finch (13), Everett, Shoemaker, and Sheppard (14), and Sumner (15).

TABLE I.

Glucose Reducing Equivalents Obtained by Various Investigators for Common Sugars.

Sugar.	Lewis-Benedict modification.						Folin-Wu method.				
	Willaman-Davison (2).	Thomas-Dutcher (10).	Greenwald-Samet-Gross (16).	Rowe-Wiener (17).	Pucher-Finch (18).	Poe-Klemme (2).	Greenwald-Samet-Gross (19).	Rowe-Wiener (20).	Pucher-Finch (3).	Everett-Shoemaker-Sheppard (3).	Poe-Klemme (3).
l-Arabinose	1.00	1.01	1.02		1.10	1.06	0.80		0.87	0.82	0.85
Xylose	1.07	1.08	1.09		1.06	1.06	0.94		1.03	1.01	1.01
Levulose	1.00	0.99	0.96	0.99	1.10	1.01	0.91	0.90	1.13	0.94	0.95
Galactose	0.88		0.95	0.85	0.93	0.93	0.75	0.77	0.75	0.75	0.74
Mannose	1.00		1.08	1.00		0.98	0.58	0.55		0.59	0.57
Lactose. H_2O	0.75		0.78	0.76	0.76	0.74	0.45	0.45	0.48	0.43	0.46
Maltose. "	0.77		0.82	0.82	0.74	0.77	0.40	0.40	0.41	0.40	0.42

Sugar.	Sumner method.				Benedict, first modification.		Folin method.	
	Greenwald-Samet-Gross (21).	Sumner (15).	Everett-Shoemaker-Sheppard (8).	Poe-Klemme (8).	Everett-Shoemaker-Sheppard (4).	Poe-Klemme (4).	Everett-Shoemaker-Sheppard (5).	Poe-Klemme (5).
l-Arabinose	1.17	1.11	1.16	1.14	0.77	0.74	0.58	0.44
Xylose	1.14	1.11	1.14	1.14	1.12	1.04	0.84	0.73
Levulose	1.00	1.00	0.98	1.01	1.15	1.26	1.02	1.28
Galactose	0.95	1.00	0.93	0.98	0.68	0.67	0.49	0.41
Mannose	0.76		0.97	0.98	0.80	0.73	0.46	0.37
Lactose. H_2O	0.70	0.81*	0.77	0.78	0.59	0.57	0.27	0.26
Maltose. "	0.70	0.81*	0.75	0.75	0.67	0.63	0.44	0.27

Figures in parentheses refer to articles describing the methods used by the different investigators.

* Anhydrous sugar used.

The results of the authors as given in Table I check those of previous investigators with the exception of a few cases. A somewhat higher result was obtained for levulose by the Benedict

copper method than was obtained by Everett, Shoemaker, and Sheppard. Their results with the new Folin method also did not coincide with ours. The value which we obtained for levulose by this method was also much higher. For the balance of the sugars we found somewhat lower results. Everett and his coworkers obtained a value of 0.27 for lactose and 0.44 for maltose against our values of 0.26 and 0.27. It appears that the latter results are the more nearly correct values since all other investigators, using other methods, have obtained about the same reducing equivalents for lactose and maltose. With the picric acid and the Folin-Wu methods Pucher and Finch obtained a higher equivalent for levulose than had been obtained by previous investigators. We failed to check the results of Pucher and Finch but did check the results of previous investigators. Pucher and Finch made thirty-three determinations on three different samples of levulose and obtained an average result of 1.10 which is over 10 per cent higher than the average of other investigators. The result for mannose with the Sumner method does not check that obtained by Greenwald and his coworkers but does check the one obtained by Everett *et al*. The former investigators, however, used the original Sumner method, while we used the latest modification.

The results recorded in the remaining part of this paper are the average of at least three determinations. In case the first three results did not check, then three additional samples were run. In all cases a preliminary determination was made with every sugar by each of the methods. In event that the readings of the standard and of the sugar were not within 10 per cent of each other, a new sample of the sugar was made up. The amount used was such that the two readings were approximately equal. This was necessary because Rothberg and Evans (22) have shown that an appreciable error will result if there is much difference in the length of the column of the color solution of the unknown and that of the standard.

The strength of the sugar solutions used varied with the method and also with the relative reducing power of the sugars employed. In general, however, for the copper methods and the Kingsbury method, amounts were used which would match the color produced by a 0.02 per cent solution of glucose. For the Lewis-Benedict modification and the Sumner method an amount neces-

sary to match the color of a 0.1 per cent solution of glucose was used.

No results have been reported with the colorimetric method recently proposed by Kingsbury (9), with 2,4-dinitro-1-naphthol-7-sulfonic acid. The same is true for the latest modifications of the Benedict (6), Folin (7), and Folin-Wu (7) methods. In Table II are listed the equivalents obtained with the common sugars by these newer methods.

Of the more recent methods listed in Table II, the lowest equivalents are given by the new Folin method, except for levulose. The results for the new Folin method compare rather closely with those of the original method. This was to be expected since only the amounts of ingredients and the methods of

TABLE II.

Glucose Reducing Equivalents for Common Sugars Obtained by More Recently Proposed Colorimetric Methods.

Sugar.	Folin, new.	Folin-Wu, new.	Benedict second modification.	Kingsbury.
l-Arabinose	0.469	0.840	0.741	1.047
Xylose	0.779	1.009	0.941	1.032
Levulose	1.139	0.950	1.223	1.005
Galactose	0.466	0.695	0.626	1.002
Mannose	0.376	0.511	0.605	1.010
Lactose. H_2O	0.246	0.450	0.444	0.728
Maltose. "	0.262	0.425	0.470	0.746

mixing were changed. There was a somewhat greater change in the ingredients of the new Folin-Wu reagents, but still the equivalents are very similar to those obtained with the original method. The results for the latest Benedict modification differ considerably from those given by the former modification listed in Table I. The values for *l*-arabinose, levulose, and galactose are about the same, while the values for the remaining sugars are lower with the latest modification. Somewhat different results were to be expected since the copper reagent differs materially. In this reagent Rochelle salt is used in lieu of sodium citrate, and sodium nitrate and alanine are new ingredients. With the Kingsbury method the pentoses and hexoses give equivalents around unity, while those of the disaccharides are much lower.

The reducing equivalents for the rarer sugars with all of the methods are reported in Table III.

With the rarer sugars listed in Table III, as well as with the more common sugars given in Tables I and II, it will be seen that the copper methods give much lower results. Of the copper methods the first modification of the Benedict method gives the highest equivalents and the original Folin method gives the lowest. The results for the other methods (Lewis-Benedict modification, Sumner, and Kingsbury) are much higher and are, in general, nearer unity. It is rather unusual to find such low results as are given by some of the rare sugars with the copper methods. There are a number of equivalents one-third and one-fourth the

TABLE III.

Glucose Reducing Equivalents for Some Rare Sugars.

Sugar.	Lewis-Benedict modification.	Folin-Wu.	Sumner.	Benedict, first modification.	Folin.	Folin, new.	Folin-Wu, new.	Kingsbury.	Benedict, second modification.
d-Arabinose	1.028	0.641	1.127	0.781	0.349	0.411	0.654	1.003	0.795
Cellobiose	0.802	0.577	0.833	0.735	0.285	0.321	0.569	0.790	0.721
Fucose	1.024	0.394	1.000	0.335	0.137	0.184	0.428	0.787	0.327
Glucoheptose	0.817	0.620	0.865	0.833	0.676	0.664	0.639	0.960	0.786
β-*d*-Glucose	0.991	1.002	1.003	1.013	1.017	0.985	1.003	1.000	1.004
Glucosamine	0.790	0.845	0.334	1.187	1.163	1.162	0.851	0.573	0.942
Rhamnose	1.101	0.366	0.975	0.389	0.144	0.222	0.406	0.804	0.310

value of glucose. For fucose and rhamnose we find equivalents one-seventh that of glucose. The values for β-*d*-glucose are in every case nearly unity, or the value of ordinary glucose.

Of the sugars listed in Table III, glucosamine has had its equivalent determined with three of the methods and rhamnose with one method. Greenwald, Samet, and Gross (11) found the following equivalents for glucosamine: picric acid method 0.98, Folin-Wu method 0.92, and Sumner method 0.34. Our values for the two former methods are much lower, being 0.790 and 0.845, respectively. Willaman and Davison (2) found the equivalent for rhamnose to be 1.298 with the picric acid method, while our result was somewhat lower, being 1.010.

SUMMARY.

1. The glucose reducing equivalents have been checked for the common sugars with the older colorimetric methods.

2. The glucose reducing equivalents have been determined for the more common sugars with the recently proposed colorimetric methods.

3. The glucose reducing equivalents have also been determined for a number of rare sugars with all of the satisfactory colorimetric methods.

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STUDIES ON INTESTINAL ABSORPTION.

I. THE ABSORPTION OF LACTIC ACID.

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Several years ago a method was established in this laboratory (1) by means of which absorption from the intestinal tract of the rat could be expressed as amount absorbed per 100 gm. of body weight per hour. Absorption of several hexoses, pentoses, and amino acids (1, 2) was studied and it was found that each substance was absorbed at a characteristic rate and that this rate was within wide limits independent of the amount and of the concentration of the substance present in the intestine. Wilson and Lewis (3) recently elaborated and confirmed the results obtained for the two amino acids, glycine and alanine, and added a study of the absorption of glutamic acid and leucine, for which the mechanism of absorption seems to be essentially the same.

When absorption of lactic acid was determined incidental to a study of glycogen formation in the liver from this substance (4), it was found that with larger amounts introduced the rate of absorption rose, which is contrary to the behavior of sugars and amino acids. Furthermore, whereas stereoisomeric sugars were absorbed at widely varying rates, *d*-, *l*-, and *dl*-lactic acid were absorbed at practically the same rate, as had already been observed by Dakin (5) in a study on isolated loops of the intestine. In rats the free acid was absorbed somewhat more slowly than the sodium salt. It will have to be shown whether this rule applies to all organic acids of a strength comparable to that of lactic acid. With the much weaker amino acids Wilson and Lewis found the same for glycine but the opposite for alanine.

EXPERIMENTAL.

Male and female rats of 125 to 152 gm. of body weight were used. They were all bred under uniform conditions in this

laboratory. Food was withheld for 24 hours prior to the experiment. A *dl*-lactic acid solution was neutralized with NaOH so that the concentration of the lactic acid amounted to 12 to 15 per cent. 2.2 cc. of the solution were introduced by stomach tube. A delivery of the same amount was made into a volumetric flask and from the determination of the lactic acid in an aliquot part, the amount fed was calculated. On an average practically the same amount of lactate was fed to the groups of rats in which absorption was determined after 1, 2, 3, and 4 hours (Table I). The rats were killed by a blow on the head. The whole intestinal tract was excised after tying the esophagus and rectum. The intestines were slit open and washed repeatedly with portions of hot water. The washings were precipitated with colloidal iron (under addition of a few drops of a saturated Na_2SO_4 solution) and the solution was made up to 500 cc.; an aliquot part of the filtrate was precipitated with $CuSO_4$ and $Ca(OH)_2$ and the filtrate was used for determinations of the lactic acid present by means of the method of Friedemann, Cotonio, and Shaffer (6). It had already been found that the colloidal iron precipitation does not lead to a loss of lactic acid (4). 2 to 4 mg. of lactic acid were present in each determination. The few animals (about 10 per cent of those fed) in which diarrhea occurred were discarded. The large amount of fluid present in the stomach, especially after the 1 and 2 hour absorption periods, made it appear possible that the solution entered the intestines at so slow a rate that absorption was limited by this factor. Lactic acid present in the stomach and the rest of the intestinal tract was therefore determined separately in a number of experiments of each absorption period. The intestinal tract of fasting rats when extracted for lactic acid yielded in four experiments 5.7, 5.8, 8.4, and 6.8 mg. or on an average 6.2 mg. per 100 gm. of rat. This amount represents the lactic acid present in the intestinal wall plus the content and was subtracted from the amount recovered in each absorption experiment. Since it had been found in previous work that the simultaneous introduction of two sugars (7) or a sugar plus an amino acid (2) lowered the rate of absorption of both substances, glucose was added to the Na lactate solution and absorption of both substances was determined. The glucose was estimated by means of the Bertrand method, as has been done in previous work on absorption of carbohydrates.

DISCUSSION.

Table I shows that an average of 55.5 mg. of lactic acid was absorbed in the 1st hour (with an average error of a single experiment of $\pm$ 6 mg.). In case of a constant rate of absorption, in 2 hours 111 mg., in 3 hours 166 mg., and in 4 hours 222 mg. should have been absorbed. The values actually observed (Table I) are considerably below those calculated for a straight line absorption; they indicate that absorption of lactic acid diminishes from hour to hour. The most probable explanation of this diminution is a dependence of the rate of absorption of lactic acid on the amount present in the intestine.

In the 1st hour 25.8 per cent of the lactic acid fed was absorbed. When it is calculated from the average values in Table I how much lactic acid is present in the intestine after 1, 2, and 3 hours and 25.8 per cent of each of these amounts is taken, it is found that in 2 hours 44.9, in 3 hours 59.1, and in 4 hours 69.7 per cent of the amount fed ought to have been absorbed if the rate of absorption were strictly proportional to the amount present in the intestines. The figures in Table I, indicating the percentage absorbed of the amount fed, are fairly close to those calculated, at any rate, much closer than they are to those calculated for a straight line absorption which would be 51.6 for the 2 hour, 77.4 for the 3 hour, and 103.2 per cent for the 4 hour absorption period.

That the rate of absorption depends on the amount present in the alimentary canal is also shown in experiments in which a much smaller amount of lactic acid was fed. When 51.8 and 53.3 mg. of lactic acid per 100 gm. of body weight were given to two rats, 23.1 and 21.9 mg. were absorbed in 1 hour, whereas in Table I from 215.8 mg. fed 55.5 mg. were absorbed, as indicated by the average value for the 1 hour absorption period.

The last two columns of Table I show the amounts of lactic acid recovered from the stomach and intestines in those experiments where separate determinations were made. The figures for the 3 and 4 hour absorption periods are chiefly of interest. Since in every case the intestines contained a considerable amount of lactic acid, slow evacuation of the stomach is not responsible for the diminishing rate of absorption. Nevertheless, this factor should not be completely neglected since it apparently can limit absorption in certain cases. In two experiments, which are not

TABLE I.

Rate of Absorption of dl-Lactic Acid Fed as Sodium Salt.

Sex.	Body weight.	Per 100 gm. of rat.		Absorbed of amount fed.	Recovered per 100 gm. of rat.	
		Fed.	Absorbed.		Stomach.	Intestine.
1 hr. absorption period.						
	gm.	*mg.*	*mg.*	*per cent*	*mg.*	*mg.*
M.	129.0	211.4	54.7	25.9		
F.	125.0	219.0	46.7	21.3		
"	137.5	212.0	50.9	24.0		
"	128.0	229.2	59.6	26.0		
"	131.0	197.1	70.7	35.8	78.3	54.3
M.	145.5	226.1	50.6	22.3	151.2	30.5
Average...	132.7	215.8	55.5±6	25.8		
2 hr. absorption period.						
F.	152.0	242.2	134.4	55.4		
"	146.0	197.8	85.2	43.1		
M.	128.0	213.0	87.6	41.1		
F.	125.5	208.0	84.8	40.8		
"	129.0	188.0	62.2	33.1		
M.	141.0	233.4	101.1	43.3	89.1	49.4
F.	134.5	213.4	102.2	47.9	55.5	61.9
Average...	136.6	213.7	93.9±16	43.5		
3 hr. absorption period.						
F.	130.0	210.5	141.7	67.3		
"	133.5	219.0	141.1	64.4		
M.	125.0	225.5	153.2	67.9		
F.	127.5	208.0	154.9	74.4		
"	138.5	208.2	130.4	62.7	68.1	15.9
"	135.0	212.6	137.4	64.6	38.4	43.0
"	138.5	185.3	94.5	51.0	59.5	37.5
"	139.0	203.7	106.7	52.4	65.2	38.0
"	134.5	210.5	108.5	51.6	72.8	35.4
Average...	133.5	209.3	129.8±18	61.8		
4 hr. absorption period.						
F.	127.0	216.0	139.0	64.3		
M.	128.0	220.7	191.2	86.6		
"	128.0	229.2	204.4	89.2		
F.	135.0	207.0	113.4	54.8	67.7	32.1
M.	140.5	214.8	167.7	78.1	24.8	28.5
F.	135.0	213.6	160.7	75.2	12.4	46.7
"	137.0	205.0	164.0	80.0	17.1	30.1
Average...	132.9	215.2	162.9±22	75.5		

included in the table, rates of absorption 50 per cent below the average were found. A separate analysis of stomach and intestines was made. In one case 7.5 and in the other 9.2 mg. of lactic acid were recovered from the intestines, values which are close to the blank figures. Slow evacuation of the stomach undoubtedly explains the unusually low rates of absorption in these two animals.

Table II gives the results of four experiments in which Na lactate plus glucose was fed and in which absorption was determined after 2 hours. The amount of lactic acid introduced was the same as in the other experiments. Absorption was slower; on an average only 66.4 instead of 93.9 mg. were absorbed per 100 gm. The three experiments in which the lactic acid remaining in the stomach and intestine was determined separately, show that the amounts present in the intestine are of about the same order as in the experiments where only lactic acid was fed. This indicates that slow emptying of the stomach was not responsible for the reduced rates of absorption of lactic acid. Glucose was also absorbed at a rate much slower than that found when only the sugar is fed (an average of 181 mg. instead of 390 mg. (8)). In the case of glucose the stomach factor is undoubtedly of importance, as is shown by the fact that in two experiments there was very little or no sugar present outside of the stomach. In one instance however, when 59 mg. of glucose were recovered from the

TABLE II.

Rate of Absorption of dl-Lactic Acid (Fed as Sodium Salt) and Glucose from a Solution of Both Substances.

Absorption period 2 hours.

Sex.	Body weight.	Fed per 100 gm. of rat.		Absorbed per 100 gm. of rat.		Recovered per 100 gm. of rat.			
						Stomach.		Intestine.	
		Lactic acid.	Glucose.	Lactic acid.	Glucose.	Lactic acid.	Glucose.	Lactic acid.	Glucose.
	gm.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
F.	137.0	218.0	495.6	70.4	167.0				
"	141.5	211.0	482.6	65.7	237.7	78.9	185.0	72.6	59.0
"	145.5	208.9	420.1	56.0	129.1	134.9	291.0	24.2	0
"	145.0	210.2	443.3	73.4	190.3	102.0	233.0	41.0	20.0
Average...	142.2	212.0	460.4	66.4	181.0				

intestine, slow evacuation of the stomach cannot have been the limiting factor for the rate of absorption. Mutual inhibition of absorption seems to occur in a mixture of lactic acid and glucose, just as it was observed to occur in mixtures of two sugars or a sugar and amino acid.

SUMMARY.

1. Absorption of *dl*-lactic acid (fed as sodium salt) from the intestinal canal of rats was determined 1, 2, 3, and 4 hours after feeding the same amount per weight. It was found that the rate of absorption decreased with time and was roughly proportional to the amount of lactic acid present in the intestine.

2. Attention was drawn to the fact that the mechanism of absorption of lactic acid differs from that observed for sugars and amino acids. With these two groups of substances the rate of absorption is within wide limits independent of the amount present in the intestine.

3. Slow evacuation of the stomach can limit the rate of absorption of lactic acid in a certain number of cases.

4. From a solution containing lactic acid plus glucose both substances were absorbed at rates below those observed when pure solutions were fed.

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STUDIES ON INTESTINAL ABSORPTION.

II. THE ABSORPTION OF ETHYL ALCOHOL.

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A method which permits the quantitative determination of the absorbing capacity of the whole intestinal tract as a physiological unit (1) is of particular advantage in a study of absorption of ethyl alcohol. Unlike sugars and amino acids (1, 2) alcohol has no fixed rate of absorption. The amount absorbed per unit of time is determined by the quantity of alcohol passing from the stomach into the small intestine. This factor which is important for absorption of alcohol in the intact animal was found when the stomach and the rest of the intestinal tract were analyzed separately for their alcohol content.

Nemser (3) studied absorption of alcohol by means of the multiple fistula method of London. He fed a definite amount of alcohol to dogs and determined the amount absorbed in different parts of the alimentary canal. He found that the jejunum absorbs the largest amount. Hanzlik and Collins (4) investigated the mechanism of absorption of ethyl alcohol from isolated intestinal loops. They observed that absorption from the small intestine of cats and dogs is practically arrested at the end of half an hour after the injection. After testing experimentally various possibilities, they came to the conclusion that the arrest of absorption is due to a retention or binding of a certain quantity of alcohol in the intestinal tissues. It will be shown later that, independent of the amount fed, a retention of a fairly constant quantity of alcohol is also observed in rats.

EXPERIMENTAL.

Male rats of 140 to 200 gm. of body weight were kept without food for 24 hours. 2.5, 4, or 5 cc. of a 10 to 40 per cent alcohol solu-

tion were fed by stomach tube. The same quantity of alcohol that was fed was delivered into a volumetric flask. 1 hour after the feeding the animals were killed and the alcohol content of the whole intestinal tract was determined. The difference between the amount fed and that recovered represents the amount absorbed. In some cases stomach and intestines were analyzed separately. The gut was cut open under water and was transferred to a 300 cc. Kjeldahl flask. After addition of water to a volume of 150 cc. about 50 per cent was distilled over. Bumping and frothing were prevented by addition of a pinch of tannic acid. The distillate was made up to a convenient volume and aliquot parts were taken for analysis. The method of Hamill (5), which is a modification of the Nicloux procedure, was followed, except that the reagents were of double strength. An alcohol solution exactly 0.1 per cent was subjected to distillation and then analyzed with the result that 98 to 99 per cent was recovered. The intestinal tract of fasting rats contained only traces of alcohol (less than 1 mg.) so that it was not necessary to apply a blank correction. In several experiments massage of the stomach through the intact abdominal wall was carried out. This was done either by gently squeezing the stomach between two fingers or by pressing it against the vertebral column and massaging it in the direction of the duodenum. It was not necessary to use strong pressure in order to achieve the desired result.

Table I is arranged according to the amount of alcohol fed. In the experiments in which 2.5 to 4.0 cc. of a 10 per cent alcohol solution were fed only a small quantity of alcohol was recovered from the intestinal tract after 1 hour; yet, this residual amount disappeared rather slowly. Rats 3 and 6 which were killed 2 hours after feeding showed only a slightly lower alcohol content than those examined after 1 hour. This confirms a similar observation of Hanzlik and Collins and stands in marked contrast to the behavior of glucose. The latter was found to disappear promptly and completely from the gut (6).

About 100 mg. per hour were absorbed when 2.5 cc. of a 10 per cent solution were fed. The absorption increased when larger amounts were given and reached a value of 450 mg. per hour when 5 cc. of a 20 per cent solution were fed. Up to this point the amount absorbed is fairly parallel to the amount fed and the con-

TABLE I.

Absorption of Ethyl Alcohol.

Rat No.	Body weight.	Fed.	Percentage of alcohol.	Per 100 gm. body weight.				
				Alcohol fed.	Alcohol recovered.			Alcohol absorbed.
					Stomach.	Intestine.	Total.	
	gm.	cc.		mg.	mg.	mg.	mg.	mg.
1	173	2.5	10	135			8	127
2	174	2.5	10	137			24	113
3*	166	2.5	10	144			7	137
4	156	4.0	10	243			49	194
5	154	4.0	10	246			32	214
6*	133	4.0	10	286			16	270
7	194	2.5	20	245			76	169
8	158	2.5	20	300			64	236
9	148	2.5	20	321			66	255
10	172	5.0	20	565			302	263
11†	173	5.0	20	568	27	36	63	505
12	167	5.0	20	584			318	266
13	162	5.0	20	611	182	51	233	378
14	155	5.0	20	633	142	26	168	465
15	155	5.0	20	647	203	32	235	412
16†	142	5.0	20	704	105	56	161	543
17†	134	5.0	20	730	40	40	80	650
18	129	5.0	20	760	269	41	310	450
19	155	2.5	40	574			326	248
20	164	2.5	40	579			343	236
21†	142	2.5	40	680	154	21	175	505
22	138	2.5	40	703	382	13	395	308
23	161	4.0	40	995			611	348
24	156	4.0	40	1050			587	463
25†	171	5.0	40	1120	286	67	353	767
26	166	5.0	40	1200			803	397
27†	164	5.0	40	1207	450	12	462	745
28	162	5.0	40	1218	750	27	777	441
29	163	5.0	40	1220	942	13	955	265
30	162	5.0	40	1225	843	26	869	356

* Killed 2 hours after feeding. All other animals were killed after 1 hour.

† Massage of stomach.

centration appears to have little influence on absorption. Hanzlik and Collins, who introduced 5 to 95 per cent solutions containing equal quantities of alcohol into isolated intestinal loops, also reached the conclusion that absorption is scarcely influenced by the concentration of alcohol. However, as shown in Table I, the feeding of 5 cc. of a 40 per cent solution did not cause a more rapid absorption than the feeding of 5 cc. of a 20 per cent solution, though the absolute amount of alcohol introduced is twice as much in the former case. This indicates that the intact animal absorbs alcohol from a 40 per cent solution less readily than from a 20 per cent solution. Since in the experiments with a 40 per cent solution all the unabsorbed alcohol was found in the stomach, paralysis of this organ and hence a diminished passage of alcohol into the small intestine seems to be mainly responsible for this finding.

The symptoms shown by the rats depend on the rate of absorption of alcohol. They varied from a slight paralysis after the feeding of 2.5 cc. of a 20 per cent solution to more or less complete insensibility after the feeding of 5 cc. of a 20 to 40 per cent solution. The pressing out of the stomach content by massage has led to death of the animals when carried out too rapidly. Animals receiving 5 cc. of a 20 per cent alcohol solution survived indefinitely, while two animals which were given 5 cc. of a 40 per cent solution died, one after 6 hours and the other after 30 hours.

In the experiments in which separate determinations were made it was found that the intestine always contained the same small quantity of alcohol (12 to 56 mg.), no matter how much alcohol was fed or how much alcohol was present in the stomach. This residual amount of alcohol, which is probably retained in the intestinal wall and which disappears very slowly, has already been commented upon. The extremely low alcohol content of the small intestine during absorption of large quantities of alcohol must be due to immediate absorption of the alcohol released by the stomach. Even when massage was applied and the stomach emptied more rapidly than normally, the residual amount of alcohol in the small intestine was not increased (except in Rat 25 which died 55 minutes after feeding). It was not possible to demonstrate an upper limit of the rate of absorption of alcohol from the small intestine in any of the experiments made, since

the animals died of alcohol intoxication before such a limit was reached. In the case of glucose and other sugars the rate of absorption cannot be increased, no matter how much sugar is introduced into the small intestine.

Massage of the stomach was applied in six experiments and in each case more alcohol was absorbed than in control experiments in which the same quantity of alcohol was fed. This indicates that absorption of alcohol in the intact animal depends on the rate at which alcohol passes from the stomach into the intestine. Rats

TABLE II.

Influence of Alcohol on Absorption of Glucose.

The animals were killed 2 hours after feeding.

Body weight.	Per 100 gm. body weight.				Blood sugar.	Body weight.	Glucose per 100 gm. body weight.		Blood sugar.
	Glucose.		Alcohol.						
	Fed.	Absorbed.	Fed.	Absorbed.			Fed.	Absorbed.	
gm.	*mg.*	*mg.*	*mg.*	*mg.*	*mg. per 100 cc.*	*gm.*	*mg.*	*mg.*	*mg. per 100 cc.*
130	730	392	162		122	128	790	512	153
133	711	448	151	110	148	137	740	577	161
136	630	372	133	116	198	128	665	519	152
130	669	585	140	129	154	115	743	453	137
138	733	420	132	101	149	124	784	410	148
121	628	559	165	149	133	130	750	432	148
140	763	337	190	151	150	140	774	458	187
133	800	329	200	160	139	126	756	570	170
133	708	430	159	131	149	128	750	491	157

25 and 27 which were subjected to massage absorbed the enormous quantity of 767 and 745 mg. of alcohol per 100 gm. of rat per hour. No other substance has as yet been found which is absorbed anywhere nearly as rapidly as alcohol. The most rapid absorption of glucose observed in this laboratory amounted to 260 mg. per 100 gm. of rat per hour and is far below the highest rate of absorption of alcohol.

Influence of Alcohol on Absorption of Glucose.

Edkins and Murray (7) observed in cats under amytal anesthesia that after the feeding of glucose plus alcohol the blood

sugar rose more rapidly, reached a higher peak, and fell more rapidly than after the feeding of glucose alone. Since alcohol alone had little effect on blood sugar, they concluded that alcohol accelerates the absorption of glucose from the alimentary canal. It may be pointed out that the blood sugar curve depends not only on the rate of absorption but also on the rate of utilization of sugar. Since alcohol has been shown to have a marked sparing action on carbohydrate oxidation (8), the higher blood sugar curve observed by Edkins and Murray after the feeding of glucose plus alcohol may have been due to this factor; *i.e.*, alcohol was oxidized in preference to glucose. In the experiments recorded in Table II unanesthetized animals were used and absorption was determined directly. An accelerating effect of small quantities of alcohol on glucose absorption could not be detected. In the presence of alcohol the individual variations in the absorption of glucose were more marked than in the control series.

SUMMARY.

1. The rate of absorption of alcohol depends on and is roughly parallel to the amount fed.

2. Up to a 20 per cent solution the concentration has little influence on the rate of absorption. From a 40 per cent solution alcohol is absorbed more slowly than from a 20 per cent solution due to slow evacuation of the stomach.

3. Separate analysis of stomach and intestine shows that the latter contains as a rule only 12 to 56 mg. of alcohol irrespective of the amount of alcohol fed or absorbed or of the amount of alcohol present in the stomach. This small residual quantity of alcohol which seems to be contained mostly in the intestinal wall disappears very slowly.

4. The fact that the small intestine always contains but this small residual amount indicates that alcohol is absorbed as rapidly as it leaves the stomach. The rate of emptying of the stomach is therefore the determining factor for the rate of absorption of alcohol.

5. Massage of the stomach which accelerates the passage of alcohol into the gut also accelerates the rate of absorption.

6. An upper limit of the rate of absorption in the small intestine cannot be determined because the animal dies of alcohol intoxication before such a limit is reached.

7. Small quantities of alcohol do not accelerate the absorption of glucose from the intestine of rats.

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THE DETERMINATION OF POTASSIUM IN BLOOD SERUM.*

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INTRODUCTION.

The existing methods (1–3) for the determination of potassium by its precipitation as the cobalti-nitrite, and subsequent use of the Ilosvay (4) modification of the familiar diazotization reaction of Greiss, have been modified to increase the speed and accuracy.

This object has been accomplished by the introduction of certain changes in the Briggs-Doisy-Bell procedure. These changes may be briefly stated as follows: (1) the introduction of the more efficient protein precipitant tungstic acid; (2) the avoiding of transfers and filtration, and the use of a new method of evaporation; (3) washing the precipitate of potassium cobalti-nitrite with 30 per cent alcohol, in which it is less soluble than in sodium chloride; (4) the use of 10 per cent acetic acid to prevent flocculation during diazotization; (5) the subjection of the standard potassium solution to the same treatment as the unknown, as suggested by Domogalla (5), with its attendant accuracy as previously shown by the author (6).

Reagents.

Standard Potassium Solutions (3).—Dissolve 382 mg. of purest potassium chloride in water and make up to 1 liter. 100 ml. of this solution contain 20 mg. of potassium. This is approximately the potassium concentration of normal blood serum.

* The expense for this work was defrayed, in part, by a gift from the Smith, Kline and French Laboratories, Philadelphia.

Doisy-Bell Cobalti-Nitrite Reagent (*3*).—Dissolve 220 gm. of *potassium-free* sodium nitrite in 400 ml. of distilled water. Dissolve 113 gm. of cobalt acetate in 300 ml. of distilled water. Mix thoroughly and add 100 ml. of glacial acetic acid. Pass a current of air through the solution for several hours, until the evolution of nitric oxide fumes ceases. Filter, stopper, and place in the ice box. It is suggested that the stock bottle remain in the ice box, small quantities being removed as required.

Sulfanilic Acid Solution.—A 0.5 per cent solution in 30 per cent acetic acid.

α-Naphthylamine Solution.—A 0.5 per cent solution in 30 per cent acetic acid.

Alcohol.—95 per cent; 30 per cent.

Sodium Hydroxide.—0.1 N.

Acetic Acid.—10 per cent.

Procedure.

Preparation of Blood Filtrate.—To 2 ml. of fresh blood serum add 14 ml. of distilled water, 2 ml. of 5 per cent sodium tungstate solution (introduced *accurately* with a 2 ml. pipette), and 2 ml. of 0.3 N sulfuric acid. Treat 2 ml. of the standard potassium solution in a like manner. The precipitation is carried out in small Erlenmeyer flasks. Shake the contents of the flasks and allow to stand for 10 minutes. Filter in dry, ashless (Whatman No. 50) filter papers; 5 ml. of the filtrate are used for each analysis.

Evaporation of Filtrate.—Transfer 5 ml. of each protein-free filtrate to separate, 15 ml. *matched*, graduated centrifuge tubes. Arrange each tube as shown in Fig. 1. The capillary tube is so arranged that the opening is 1 cm. above the level of the liquid in the tube. It is drawn out so that, by gentle suction, a partial vacuum is maintained, and at the same time the current of air enters with sufficient force to agitate the contents of the tube.

The current of air is adjusted by regulating the vacuum so that no bumping occurs. The tubes are hung in a large beaker of distilled water, kept at the boiling point by means of an electric hot plate. The use of distilled water keeps the contents of the water bath visible at all times. This type of water bath enables one to observe the process of evaporation and to adjust the vacuum as required.

Evaporation is continued to dryness, which requires about 30 minutes.

Precipitation.—To the residue obtained from the above evaporation add 1 ml. of distilled water, dissolving the residue with heating, if necessary. Add 1 ml. of 95 per cent redistilled alcohol and mix thoroughly by shaking. The precipitation is carried out by the addition of 1 ml. of Doisy-Bell cobalti-nitrite reagent. After

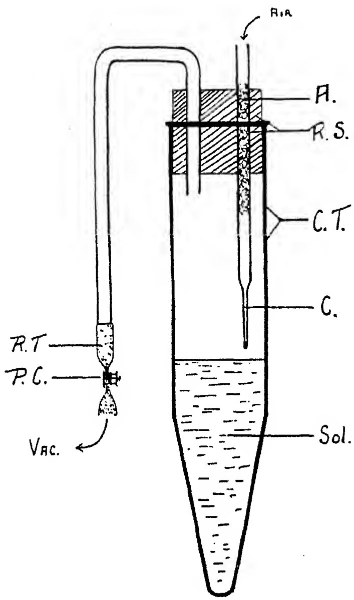

FIG. 1. Evaporation apparatus. P. C. = screw clamp; R. T. = rubber tubing; R. S. = rubber stopper; C. T. = centrifuge tube; A. = absorbent cotton; C. = capillary tube.

thoroughly mixing the contents, the tubes are set aside for 2 hours, after which they are centrifuged at 1800 to 2000 revolutions per minute for 10 minutes.

Washing.—The supernatant liquid above the precipitate is rapidly decanted. Add 3 ml. of 30 per cent alcohol, a small amount at a time, *thoroughly suspending the precipitate on each addition.* Centrifuge rapidly for 5 minutes and decant off the

alcohol. *Five* such washings are required. The use of alcohol solutions greater than 30 per cent must be avoided, since they cause precipitation of some sodium cobalti-nitrite.

After draining from the final washing with alcohol dissolve the precipitate in 0.1 N sodium hydroxide and transfer to 100 ml. volumetric flasks, using 20 ml. of the sodium hydroxide for the combined operations of washing and transferring. The sodium hydroxide is added in four 5 ml. portions. The actual procedure for this step has been found to be so important that it is given here in detail. A few drops of the first 5 ml. portion of 0.1 N sodium hydroxide are added to the precipitate which is completely suspended by tapping the tubes against the palm of the hand. The remainder of the first portion is added, 1 ml. at a time, the precipitate being kept suspended. Most of the precipitate is dissolved in the course of this treatment. This portion is then rapidly decanted into 100 ml. volumetric flasks. The second 5 ml. of sodium hydroxide are added in the same manner. At the conclusion of the transfer no trace of precipitate should be present in the tubes. The flasks are then thoroughly shaken and the tubes washed into them by a stream of water from a wash bottle, with 50 ml. of water. After being shaken thoroughly again, so that all the precipitate is dissolved, the contents of the flasks are diluted to the 100 ml. mark. The flasks should be kept tightly stoppered with ground glass stoppers to prevent oxidation of the dissolved nitrite. The flasks are then set aside for 1 hour.

Diazotization.—Transfer 5 ml. of the sodium hydroxide solution from each of the 100 ml. flasks, by pipettes, to 50 ml. volumetric flasks and dilute to 4 times their volume with 10 per cent acetic acid. Add 2 ml. of sulfanilic acid and 1 ml. of α-naphthylamine solution and again shake thoroughly. Dilute to the mark with 10 per cent acetic acid and mix by inverting the flasks several times. Allow the color to develop for 10 minutes and compare in the colorimeter. The standard solution is set at 20, and with a potassium solution containing originally 20 mg. per cent of potassium the calculation is:

$$\frac{\text{Standard}}{\text{Reading}} \times 20 = \text{mg. per cent potassium}$$

TABLE I.

Potassium Values of Serum of Some Normal and Pathological Individuals.

Patient.	Date.	Diagnosis.	K per 100 cc. blood.
	1929		*mg.*
E. S.	Nov. 22	Normal.	22.0
"	" 25	"	20.0
"	" 25	"	19.9
A. L.	" 24	"	24.0
A. E. C.	" 26	"	22.2
	1930		
I. N.	Jan. 4	"	22.2
M. B.	" 4	"	21.9
	1929		
A. S.	Nov. 25	Infectious arthritis.	13.6
"	" 25	" "	13.4
M. R.	" 26	" "	17.7
S. W.	" 30	" "	17.0
F. L.	Dec. 2	Pernicious anemia.	25.0
Fo.	" 2	" "	21.6
Sul.	" 2	" "	28.5
Zu.	" 2	Myxedema.	17.4
Cu.	" 2	Pernicious anemia.	25.2
Wa.	Nov. 30	" "	26.6
Wi.	" 2	" "	23.6
Jo.	" 30	" "	17.0

TABLE II.

Potassium in Standard Solutions.

K present.	K found.*	Error.
mg. per 100 ml.	*mg. per 100 ml.*	*per cent*
30	30	±0.0
30	30.7	+2.3
20	20.3	+1.5
20	19.7	−1.5
10	10.2	+2
10	10.0	±0.0

* Standard, 20 mg. per 100 ml.

Experimental Results.

Experimental results showing the range of potassium values in normal and certain pathological conditions are given in Table I. Table II shows the amount of potassium obtained from various concentrations of potassium in aqueous solution. The accuracy and duplication have been found to be high. The recoveries obtained from standard solutions were within 3 per cent of the true value.

The method is equally applicable to whole blood and blood plasma. In the first case ashing must precede the determination and evaporation be omitted. In the case of plasma the procedure may be carried through in the same way as for serum potassium, with heparin as the anticoagulant.

It is essential in serum potassium determination that hemolysis be prevented and the cells centrifuged out as rapidly as possible after the blood is drawn. The presence of hemolyzed red cells invariably causes high results.

SUMMARY.

1. The method for determination of potassium in blood sera by the indirect method is modified as given above, with an improvement in the speed and accuracy.

2. Data are presented showing the limits of accuracy and the extent of duplication of the method.

3. The potassium values obtained in a few normal and pathological conditions are given.

The author acknowledges the assistance afforded him by his colleague, Dr. A. G. Young of this laboratory, in providing technical assistance.

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DIETHYLBARBITURATE BUFFER.

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In the series of buffers now used, there is no other buffer system to cover the range around pH = 8 with a sufficient reliability except for the borate buffer of Sörensen. This buffer has, however, certain disadvantages which are strongly felt in practical use. The borate buffer cannot be used in compounds containing two or more hydroxyl groups, such as polyphenols, polyhydric alcohols, and especially carbohydrates of any kind. These substances combine with boric acid to form complex acids of an appreciable strength. Not only is the pH of the borate buffer perceptibly shifted in the presence of those substances but, what is worse, those substances themselves are no longer present in their original state in contact with the borate buffer. This disadvantage was encountered on the occasion of the investigation of the effect of pH on the activity of all carbohydrate-splitting enzymes and on the measurement of oxidation-reduction potentials of certain dyestuffs containing two hydroxyl groups, to be described in a subsequent paper. This gap in the buffer series is due to the fact that practically no acid was known with a dissociation constant around 10^{-8}. Recently Kolthoff (1) found a dissociation constant of approximately 10^{-8} for diethylbarbituric acid (veronal). So it seemed likely to me that this acid might be useful as a buffer for the wanted range. This turned out to be true. The following data, calculated from the experimental calibration with the hydrogen electrode, will suffice for practical purposes.

10.30 gm. of sodium diethylbarbiturate (veronal sodium) are dissolved in CO_2-free water to a volume of 500 cc. The quality of the commercial preparation was satisfactory without recrystalliza-

tion. It may be dried at 100° but loses only traces of moisture by this process. 10 cc. of this solution when titrated against 0.1 M HCl should use up accurately 10 cc. of the acid to the turning point of methyl red. If the veronal solution turns out to be a little weaker (say 1 per cent) a correction for the number of cc. indicated in the first column of Table I may be applied. The error, on neglecting this correction, however is practically negligible except for the range of pH < 7.2. When n cc. of this stock solution of veronal sodium (first column of Table I) are mixed with $(10 - n)$ cc. of 0.1 M HCl, the pH indicated in the second column

TABLE I.

Veronal Buffer.

When n cc. of 0.1 M veronal sodium are mixed with $10 - n$ cc. of 0.1 M HCl, the following pH values are obtained.

n	pH	n	pH	n	pH
(5.10)	(6.40)	6.15	7.60	9.08	8.80
(5.14)	(6.60)	6.62	7.80	9.36	9.00
5.22	6.80	7.16	8.00	9.52	9.20
5.36	7.00	7.69	8.20	9.74	9.40
5.54	7.20	8.23	8.40	9.85	9.60
5.81	7.40	8.71	8.60	(9.93)	(9.80)

The values in parentheses cannot be considered as accurately reproducible with respect to pH.

is obtained. The potentiometric calibration has been made for 25° ± 0.05°, the pH of standard acetate being considered 4.62.

This buffer has a satisfactory buffer capacity between pH 7.0 to pH 9.4, and so covers not only the gap between the phosphate and the glycocoll buffers but it also duplicates the pH range of particular physiological importance around 7.4 for which only the phosphate buffer was available till now, except for the occasional and restricted use of the cacodylate buffer (2). Though the pH range around 7.4 does not lie quite in the region of the maximum buffering capacity of this buffer, which is pH 8 to pH 9, it can be safely used between pH 7 and pH 8 with carefully prepared stock solutions.

SUMMARY.

Veronal is recommended as a buffer covering a range from pH 6.8 to pH 9.6. It replaces not only the borate buffer which is undesirable in many cases but also duplicates a part of the phosphate buffer range, especially around the physiologically important pH 7.4.

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THE LACK OF RELATIONSHIP BETWEEN THE DEVELOPMENT AND CURE OF RICKETS AND THE INORGANIC PHOSPHORUS CONCENTRATION OF THE BLOOD.

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During the past few years there has been an increasing tendency to regard the decrease of inorganic phosphorus in the blood as the fundamental phenomenon of rickets, in animals as well as in infants. The diminution is looked upon as an essential and integral part of the disorder and by many is considered the final criterion as to whether or not active rickets is present. This point of view is due largely to the fact that rickets has been studied more and more in the laboratory and less in the clinic and, furthermore, that the newer clinical studies of infantile rickets have concerned themselves more particularly with the laboratory manifestations of the disorder, such as the chemical examination of the blood. It has thus come about that the invariability of a diminished concentration of inorganic phosphorus has been accepted and become firmly established without an effort having been made to ascertain whether it is a constant phenomenon.

In 1922, in a study of the significance of clinical, radiographic, and chemical examinations in the diagnosis and incidence of rickets (1) it was pointed by Hess and Unger that although the concentration of inorganic phosphorus tended to diminish early in rickets, the disorder undoubtedly was present at times in spite of the fact that the inorganic phosphorus maintained its normal level. In the monograph on rickets, published recently by one of us (2), the matter was stated as follows: "The chemical examination of the blood is not an infallible criterion in infants. The tendency has been to overrate its value and more especially its significance." It may be added that in the peculiar form of this disorder which is

termed "renal rickets" the inorganic phosphorus is frequently above rather than below normal. It is also of interest in this connection that fractures often fail to heal although the inorganic phosphorus of the blood is at the normal concentration.

Some years ago it was reported from this laboratory that when rats were given a rickets-producing ration in which 10 per cent of dry milk was substituted for an equivalent amount of flour in the Sherman-Pappenheimer diet, although rickets was regularly induced, it was often associated with concentrations of inorganic phosphorus in the blood as high as 6.5 or 7 mg. In other papers bearing on this subject, it was noted that when 20 cc. of fluid cow's milk or of irradiated skim milk were added to the standard ration, the same lack of conformity in the phosphate curve was observed. On the other hand, when the Sherman-Pappenheimer[1] ration is irradiated, although rickets is prevented, the inorganic phosphorus may fall to the rachitic level.

Last year we referred to the fact that in some instances when inadequate amounts of antirachitic agents, for example of irradiated ergosterol or of poorly irradiated dry milk, had been given to infants as a preventive measure and when mild rickets resulted, as evinced by the Roentgen rays and careful clinical examinations, concentrations of 6 mg. or more of inorganic phosphorus and of 10 mg. or more of calcium were found; in other words, normal phosphorus values persisted in the blood (3). Incidentally, it may be added that in these cases the product of the calcium figure multiplied by the phosphorus figure, which is considered by many to be of significance in the diagnosis of rickets, was also normal or even well above the normal level in many instances.

In a preliminary communication recently published by Hess and Supplee and their collaborators (4), it was shown in curative experiments that when inadequate amounts of irradiated ergosterol were given to rachitic rats, the phosphate concentration of the blood was raised to the normal level in spite of the fact that no antirachitic or calcifying action was demonstrable in the epiphyses of the long bones. The rats used for these experiments were about 4 weeks old, weighed approximately 50 gm., and had been fed the Steenbock rickets-producing diet[2] as the basic ration. The irradiated ergos-

[1] Sherman, H. C., and Pappenheimer, A. M., *J. Exp. Med.*, **34**, 189 (1921).
[2] Steenbock, H., and Black, A., *J. Biol. Chem.*, **64**, 263 (1925).

terol was dissolved in ether and this solution was impregnated in a weighed amount of non-irradiated dry milk, after which the ether was evaporated. A similar method was resorted to when cod liver oil was used. The mixtures were prepared every 3rd day and the dry milk was reconstituted daily. At least four animals were used for each group and the phosphorus determined in the blood of each animal after a test period of 10 days. The humeri were used for the ash determinations. Table I gives a survey of one of the

TABLE I.

Lack of Relationship between Healing of Rickets and Inorganic Phosphorus of Blood and Bone Ash. Ration Containing Dry Milk (Hess and Supplee (4)).

Rickets-producing ration and supplement.	Bone ash, per cent.			Blood inorganic P, mg.			Rickets. Line test.
	Maximal.	Minimal.	Average.	Maximal.	Minimal.	Average.	
Basal ration only.................	30.19	26.37	23.29				–
10 cc. non-irradiated dry milk.....	35.41	33.47	34.44	2.96	1.90	2.26	–
10 " irradiated dry milk..........	38.27	33.83	36.39	1.77	1.14	1.14	++
10 " non-irradiated dry milk plus:							
3 mg. cod liver oil.............	39.03	34.10	36.89	2.56	0.15	0.87	+
5 " " " "	39.81	34.84	38.09	2.39	0.39	0.90	+
7 " " " "	37.58	33.41	36.65	4.81	3.40	4.21	++
10 " " " "	41.00	33.64	37.54	2.80	0.19	1.68	+++
20 " " " "	41.18	34.35	38.77	6.23	3.27	4.21	+++±
30 " " " "	41.42	37.45	40.12	4.12	0.19	2.12	+++±
Irradiated ergosterol*	32.92	29.60	31.49	7.54	4.64	6.05	–
" " †..........	34.36	27.44	30.50	6.38	3.01	4.95	–(?)

* About one-twentieth the minimal protective dose daily.

† About one-eightieth the minimal protective dose daily.

series of tests which comprised not only curative but preventive experiments. It is at once evident that a marked inconsistency prevailed between the inorganic phosphorus of the blood and the healing of the rachitic lesion, as judged by the line test, and furthermore that the percentage of bone ash was low and tended to conform to the line test rather than to the chemical analyses of the serum. For example, in one test there was absolute lack of healing associated with a bone ash of only 31.49 per cent and nevertheless the inorganic phosphorus in the blood was found to have risen to a

concentration of 6.05. These rats had been given only about one-twentieth of the minimal protective amount of a commercial solution of irradiated ergosterol. Of course, when full potencies are given, complete protection or cure is accomplished. It should be emphasized that this peculiar result, this non-conformity between the classical chemical alteration of the blood and the healing process, was due largely to the fact that the rickets-producing ration contained a considerable percentage of milk, in other words of Ca and P in a favorable ratio. Had any of the standard rickets-producing rations been employed, minimal amounts of irradiated ergosterol would not have raised the phosphorus in this way. The thousands of routine tests of irradiated ergosterol which, during the past 2 or 3 years, have been carried out in many laboratories, must indicate that such is the case.

A result of this kind emphasizes the fact that the healing of rickets is not merely contingent on an adequate percentage of phosphorus in the blood, but that an additional factor must be involved in the calcification of the rachitic cartilage. It suggests a local factor in the pathogenesis and healing of rickets. It indicates also a peculiar tendency of irradiated ergosterol to raise the phosphate concentration of the blood, quite apart from its antirachitic or calcifying activity. It will be noted in Table I that in some of the tests cod liver oil rather than irradiated ergosterol was added to the ration. These additions brought about healing which varied in degree according to the amount of oil which was given. It is of significance that the healing which was brought about by means of cod liver oil was accompanied by only a moderate rise in the inorganic phosphorus of the blood and that the concentrations attained were below those which were found when irradiated ergosterol had been given and when the line test had been negative. Associated with this healing brought about by cod liver oil was an increase in the percentage of the ash of the long bones.

Another type of curative experiment was carried out and has been described in a preliminary note (5). In this series a form of extreme rickets was induced by means of a diet which contained a very marked excess of calcium. This increased ratio of calcium over phosphorus was accomplished by substituting yellow corn-meal for the yellow corn in the standard Steenbock rickets-producing ration. By this means the ratio of Ca:P was rendered excep-

TABLE II.

Lack of Relationship between Healing of Rickets and Inorganic Phosphorus of Blood. Very Low Phosphorus, High Calcium Ration.

Weight of rat.	Diet.	Supplement.	Healing, radiologic.	Serum. Ca	Serum. P
gm.				*mg.*	*mg.*
50–54	Ca:P ratio 11.5:1.	Cod liver oil. 300 mg.	None.	15.7	5.5
46–60			"		
50–62			Trace.		
50–54			"		
60–60			None.	13.8	6.2
74–80			"		
60–74			"		
70–70			Trace.		
50–54		100 mg.	None.	14.9	3.8
50–54			"		
56–60			Slight.		
70–64			Moderate.		
60–60		25 mg.	None.	15.7	3.4
60–80			Trace.		
64–64			"		
70–70			"		
50–56		10 mg.	None.	11.2	3.7
40–34			"		
60–64			"		
50–40			"		
64–84		Viosterol* (irradiated ergosterol). 0.1 cc.	None.	12.1	3.6
54–54			"		
60–74			"		
80–80			Trace.		
66–70		0.001 cc.	None.	11.9	4.1
74–80			"		
66–74			Trace.		
60–74			Very slight.		
50–50			None.	11.0	6.7
50–60			Trace.		
50–60			Slight.		

TABLE II—*Concluded.*

Weight of rat.	Diet.	Supplement.	Healing, radiologic.	Serum.	
				Ca	P
gm.				*mg.*	*mg.*
64–70			None.	9.8	3.4
54–70			"		
54–70			"		
56–60			None.	11.7	3.4
54–50			"		
52–56			"		
60–63			"		

* 100 times the potency of cod liver oil.

tionally high, varying from about 9.5 to 11.5 of Ca to 1 of P. This increased ratio was due to the low phosphorus content of the ration which contained about 0.128 per cent P instead of about double this percentage. This diminution of phosphorus was brought about by the lack of pericarp and germ in the corn-meal as compared to the corn. The ration had an excess of base over acid equivalent to about 520 cc. of 0.1 N alkali. When this ration was fed to numerous series of rats, it was found that, in spite of very large amounts of cod liver oil or of irradiated ergosterol, the rachitic lesions in the epiphyses remained unaffected or gave evidence of but slight healing, whereas the blood showed normal or even excessive concentrations of inorganic phosphorus and of calcium. In some instances the ratio was normal; in others the one or the other element was disproportionally high (Table II). In many cases in which rickets existed under these conditions, the product of the concentration of Ca $\times$ P was above rather than below normal. It has been held that if this product is as high as 45, rickets will not develop. In some of our animals it was as high as 80 or 90 and nevertheless rickets persisted. In general, it may be stated that although this product may serve as a valuable clinical guide, there are notable exceptions to the rule, both in clinical and experimental rickets, so that it must be regarded as a convenient guide rather than as a phenomenon which incorporates a principle inherent in the pathogenesis of rickets.

In this set of animals the presence of rickets was diagnosed by

the radiographic picture and the occurrence of healing was determined by the same means as well as by histologic examination. In many animals these data were further substantiated by analyses of the bones. It was found that the bones of these rats contained approximately 30 per cent instead of the normal percentage of ash, which is about 50 per cent. As stated, these lesions, which were induced by an extreme disproportion in the diet, were extraordinarily resistant to treatment with specific antirachitic measures. It will be noted in Table II that healing could not be brought about by cod liver oil even when 300 mg. were given daily, which is 40 times the curative dose. Various standard brands of cod liver oil were used. The yolk of egg likewise was ineffective in inducing healing. Furthermore, irradiated ergosterol failed to exert a curative action even when 20,000 times the minimal curative dose was fed daily. When very large amounts of irradiated ergosterol were fed, the percentage of ash in the bones was lower than when smaller quantities were given. This result is in keeping with an observation which we made not long ago (6) to the effect that irradiated ergosterol, when given with a diet deficient in calcium, tends to withdraw calcium and phosphorus from the bones. A similar reaction has been well shown in a recent paper by Brown and Shohl (7) who refer to the demineralization of the skeleton of rats after feeding toxic amounts of irradiated ergosterol. This same paradoxical reaction was found to hold true when very large amounts of cod liver oil, 300 mg. daily, were fed. It may be noted in Table II that in one of these tests not only was the inorganic phosphorus normal, but its ratio to calcium was likewise normal (13.8 to 6.2 mg.). Ultra-violet irradiation was also ineffective in bringing about healing. For this purpose, monochromatic rays were used; in one experiment a wave-length of 2967 Ångstrom units of high intensity was employed, which is highly effective when standard rations are given.

In this series of experiments the usual test period for feeding the supplements was 9 days. It was found that if this period was extended to 18 days, a definite healing effect could be noted, although the concentration of inorganic phosphorus in the blood had not risen to a much higher level than when only 9 days were allowed to elapse.

In addition to its significance in relation to blood phosphorus,

this experiment illustrates that a state of rickets can be brought about which is resistant to thousands of times the minimal curative dose of various rachitic agents. It suggests differences in intensity in standard rachitic rations and how such differences may markedly affect the dosage and titration of antirachitic agents. This fact is not taken into consideration sufficiently by laboratory workers and leads to difficulties in interpreting the comparative potency of preparations of cod liver oil and of irradiated ergosterol. As a rule the rations which are fed have grain as their chief components. It is taken for granted that the composition of the rations is constant and that the various ingredients are present in an invariable proportion. As a matter of fact, it has frequently been shown that the content, for example of phosphorus, varies considerably according to whether the grain was grown in one part of the country or another and also according to the time of the year when it was harvested. At its best, the biological test is not exact, and its inherent weaknesses should not be increased by lapses in technique which can be controlled and rectified.

As mentioned, in addition to the question of the relation of inorganic phosphorus to the development or cure of rickets, there is another aspect which is raised by these observations. The established fact that the inorganic phosphorus of the blood is generally diminished in rickets has led to the inference that the disturbance associated with this disorder is of a systemic nature and that local factors play no part in its pathogenesis. For some years we have not been entirely of this opinion, believing that proof of a systemic factor did not necessarily rule out the participation of a local disturbance. Several years ago one of us showed that in infants rickets could not be prevented or cured by giving as much as 4 gm. daily of sodium phosphate. More recent experiences of this kind have given similar results; the phosphate in the blood is raised temporarily, but soon falls to the original level. Many years ago Grosser (8) showed that the retention of phosphorus could not be appreciably increased in rachitic infants merely by injections of solutions of sodium phosphate. Heymann (9) has extended these observations and come to a similar conclusion. It seems to us that these observations, considered in conjunction with our experiments, indicate that there is probably a local as well as a systemic factor which prevents the calcification of the

epiphyses in rickets—something which is lacking in order to bring about a binding of the calcium and phosphorus. Although the experiment with the very high calcium ration may not be conclusive on account of the artificial nature of the diet, it would seem that the tests with the ration including milk, both preventive and curative, are not open to this criticism and must be considered strong evidence in favor of a local factor in connection with the development and the cure of rickets. This diet is more nearly comparable to that of infants on account of its exceptional content of phosphorus as well as of calcium.

SUMMARY.

Rickets may be associated with normal concentrations of inorganic phosphorus in the blood.

If milk is incorporated in a standard rickets-producing ration, a peculiar chain of circumstances can be brought about. On the addition of small amounts of irradiated ergosterol, the inorganic phosphorus is raised to the normal level without accompanying signs of healing of the rachitic lesion. Animals which receive supplements of cod liver oil do not attain as high a concentration of phosphorus but do show definite evidences of healing. Of course, if full doses of irradiated ergosterol are given, prevention or cure can be relied upon. Irradiation may elaborate a factor which increases the inorganic phosphorus in the blood, quite apart from any antirachitic action.

This paradoxical phenomenon—a rise of inorganic phosphorus unaccompanied by a healing of the epiphyses—shows that although rickets usually is associated with a decrease of the inorganic phosphorus of the blood, this diminution cannot be regarded as an essential or inherent feature in the pathogenesis or the healing of this disorder.

In addition to the systemic disturbance which is characteristic of rickets, there is probably a local disturbance at the epiphyses which prevents the anchorage of calcium and phosphorus in the cartilage and in the bones.

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THE DEPLETION OF MUSCLE SUGAR BY ADRENALIN.

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INTRODUCTION.

Sahyun and Alsberg (1) recently found that the decrease in blood sugar of blood incubated with muscle deglycogenated by adrenalin was greater than for the same blood incubated with normal muscle. They observed also a decrease in glucose when glucose solution was substituted for blood, and concluded that "deglycogenated whole skeletal muscle has the power of utilizing glusose *in vitro*." These authors failed to consider the glucose content of the muscle used in their experiment. This appeared to us to be a factor of considerable importance, since they used approximately equal quantities of blood and muscle in their experiments. It occurred to us that the decrease in sugar observed for deglycogenated muscle could be accounted for by a low muscle sugar for the deglycogenated muscle simply on the basis of diffusion, and need not indicate an *in vitro* utilization of glucose by the muscle. The latest work on the glucose content of muscle indicates that for the fasting animal the blood sugar and muscle sugar are approximately the same (2). Palmer (3) studied the action of adrenalin and found that after $1\frac{1}{2}$ hours the muscle sugar though lower than the blood sugar was at a hyperglycemic level. In the experiments of Sahyun and Alsberg the muscle was taken after the adrenalin had reacted for a much longer time, even after 24 hours. After considering the data available in the literature, we were tempted to determine the glucose content of muscle 24 hours after the administration of adrenalin. A few preliminary experiments showed that the reducing power of extracts of muscle of rabbits, which had been subjected to adrenalin action for 24 hours, was considerably less than for normal muscle treated

in exactly the same manner. These results indicated that the time factor was of considerable importance in the action of adrenalin upon the muscle sugar, and that the muscle and blood sugar did not run parallel after the initial period, as has been tacitly assumed by those working with adrenalin.

Plan.

The glucose content of the leg muscle of the rabbit was determined $1\frac{1}{2}$, 3, 8, and 24 hours after the subcutaneous administration of 0.25 mg. of adrenalin per kilo. The determinations were carried out on groups of litter mates, three or four at a time, one animal always serving as a control in order to determine the normal muscle glucose content of the animal.

Four experiments were planned in order to answer several pertinent questions in regard to the *in vitro* experiments of Sahyun and Alsberg. Water was substituted for the glucose and blood in the experiments of these authors to determine to what extent the diffusion of the muscle glucose into a surrounding aqueous solution took place. Sulfuric acid was substituted to eliminate the possibility of enzyme activity. Complete glycogen analyses were made to eliminate the possibility of glucose being transformed to glycogen.

Experimental Procedure.

Determination of Muscle Sugar.—The animals were killed by a blow on the back of the neck, the gastrocnemius and gluteus muscles of the right leg immediately removed, and 50 gm. of muscle plunged as quickly as possible into 75 cc. of boiling water. Boiling was continued for 15 minutes, when the liquid was decanted into a 250 cc. flask. The muscle was next cut into bits with a pair of scissors, then ground to a pulp in a mortar, and covered with 50 cc. of hot water. This mixture was allowed to stand 1 hour with occasional stirring and the liquid then decanted into the volumetric flask. The extraction was repeated twice. The combined extract was finally diluted to 250 cc. Since the determination of muscle sugar is complicated by the high content in muscle of other reducing substances, notably creatinine, we compared several muscle sugar methods described in the literature. Three different methods of precipitation were used. The first

method was according to Hoagland (4) with modifications. To 40 cc. of the filtrate in a 50 cc. volumetric flask 10 gm. of phosphotungstic acid were added with shaking until all was dissolved. The contents were made up to the mark and filtered into a 50 cc. centrifuge tube. Potassium chloride was added to precipitate the excess phosphotungstic acid. The mixture was thoroughly shaken and centrifuged. 20 cc. of the clear supernatant fluid were

TABLE I.

Comparison of Muscle Sugar Methods.

The results are expressed in mg. per 100 gm.

Method.		Control rabbits.				Adrenalinized rabbits.							
Precipitation.	Sugar determination.					1.5 hrs.		3 hrs.		8 hrs.		24 hrs.	
I. Phosphotungstic acid.	Shaffer-Hartmann.	95	77	87	86	144	134	117	101	Too low to read.	45	47	42
II. Colloidal iron and Lloyd's reagent.	Shaffer-Hartmann.			76	100	143	143	138			57		
	Folin-Wu.		54	52	58	112	109	102	64	27	33	49	
III a. Folin-Wu.	Shaffer-Hartmann.	148		124	133	201	189	152	141		88		97
	Folin-Wu.	94	82		88	95	122	122	90	50	68	72	47
III b. Folin-Wu and Lloyd's reagent.	Shaffer-Hartmann.			81	96	133	122	115	115		37		
	Folin-Wu.			62	59	99	106	68	46		20		

neutralized in a 25 cc. flask, and the blood sugar determined by the Shaffer-Hartmann method. The Folin-Wu method was tried but the blue color faded very quickly after dilution. The second filtrate was precipitated according to Palmer's method (3). To 30 cc. of the filtrate in a 50 cc. flask, 12 cc. of 5 per cent colloidal iron were added and 5 cc. of 40 per cent Na_2SO_4, and the flask placed on a water bath for 1 hour. The solution was then filtered, and Lloyd's reagent was added to the filtrate, according to Cori

and Cori (2). This filtrate gave duplicable results both by the Shaffer-Hartmann and the Folin-Wu methods. The latter gave lower results. The third precipitation was carried out by the Folin-Wu blood method (5) except that it was necessary to increase the proportion of $\frac{2}{3}$ N sulfuric acid to tungstate before complete precipitation was effected. To 30 cc. of filtrate in a 50 cc. volumetric flask, 3 cc. of 10 per cent sodium tungstate were added and 11 cc. of $\frac{2}{3}$ N H_2SO_4. The mixture was allowed to stand, then filtered. 20 cc. were neutralized in a 25 cc. flask, and sugar run both by the Shaffer-Hartmann and Folin-Wu methods. The latter again gave lower results. To the remainder of the filtrate 1 gm. of Lloyd's reagent was added, the contents shaken 2 minutes, filtered, and neutralized. Sugar was again determined by the two methods, the difference between the results obtained with and without Lloyd's reagent representing roughly the creatinine content of the muscle. In Table I is a summary of the results thus obtained.

The Shaffer-Hartmann sugar method gave more consistent results with the filtrates from the different methods of precipitation than did the Folin-Wu. Added sugar could be quantitatively recovered from the phosphotungstic acid filtrate by the Shaffer-Hartmann method. This was the method selected for comparative studies of the muscle sugar following adrenalin administration. The Folin-Wu method could not be used for phosphotungstic acid filtrates because of rapid fading of the final blue color. Sugar added to phosphotungstic acid filtrates was not quantitatively recovered. It should be noted that the Folin-Wu sugar method used with Folin-Wu filtrates gave values close to those obtained by the Shaffer-Hartmann sugar method with phosphotungstic acid filtrates. It is necessary to bear this in mind, since Sahyun and Alsberg used this method for their *in vitro* experiments.

Relating to Experiments of Sahyun and Alsberg.

Experiment 1.—Two rabbits were fasted 24 hours. One rabbit was given 0.25 mg. of adrenalin per kilo subcutaneously, the dose being repeated 4 hours later. The other rabbit served as a control. 24 hours later both rabbits were killed. 15 gm. of minced and 15 gm. of unminced muscle of each were placed in 15 gm. of water for 30 minutes. The reducing power of the solution was

then determined by the sugar method of Folin and Wu after removal of proteins by the Folin-Wu tungstate method. While the blood sugar values of the adrenalin rabbit and control rabbit were 154 and 105 respectively, the reducing powers of the aqueous muscle extracts were in the reverse order, 15 to 22 for the unminced muscle, 21 to 35 for the minced muscle.

Experiment 2.—The conditions of Experiment 1 were duplicated with the exception that $\frac{2}{3}$ N H_2SO_4 was substituted for water, in order to inhibit possible enzyme action. In this experiment the control muscle filtrate showed 3 times the reducing power of the adrenalinized muscle filtrate.

Experiment 3.—The conditions of Experiment 1 were duplicated with the exception that rabbits which had suffered deglycogenation through synthalin were used and one dose only of adrenalin was given. The animals were killed 6 hours after the dose of adrenalin. Glycogen determinations of the muscle showed less than 0.02 per cent glycogen. The control muscle showed twice the reducing power of the adrenalinized muscle filtrate.

Experiment 4.—The conditions of Sahyun and Alsberg were duplicated. The rabbits were killed 24 hours after the dose of adrenalin. Equal weights of muscle and blood were used. Glucose had previously been added to the blood so that the resulting blood sugar value was 186 mg. per 100 cc. Blood sugar, muscle sugar, and muscle glycogen were determined at death and at the termination of the *in vitro* incubation period.

Results of Experiment 4.

The results are expressed in mg. per 100 cc. of blood, per 100 gm. of muscle.

Values at death.	Control.	Adrenalin.	*In vitro.*	Control.	Adrenalin.
Blood sugar	94	95	Initial blood sugar	186	186
Muscle "	95	42	Final " "	127	91
" glycogen	166	75	" muscle glycogen	0	0
			" blood "	0	0

DISCUSSION.

The results of the analyses of the glucose content of muscle taken at various time intervals after the subcutaneous adminis-

tration of 0.25 mg. of adrenalin per kilo are so clear cut that very little discussion is necessary. The figures are given in Table II. The blood sugar and muscle sugar of the fasting rabbit are approximately the same. $1\frac{1}{2}$ hours after adrenalin administration the blood sugar rises to unusual heights. The muscle sugar though lower is at a hyperglycemic level. At 3 hours the blood sugar has risen still more, but the muscle sugar is only slightly

TABLE II.

Effect of 0.25 Mg. of Adrenalin per Kilo upon Blood and Muscle Sugar of Rabbit.

Time after adrenalin.	Glucose content, mg. per 100 cc. blood, 100 gm. muscle.					
	Sugar from:	Series I.	Series II.	Series III.	Series IV.	Average.
hrs.						
Control.	Blood.	94	98	84	106	95
	Muscle.	95	77	87	86	81
$1\frac{1}{2}$	Blood.				306, 256	281
	Muscle.				144, 134	139
3	Blood.			320	308	314
	Muscle.			117	101	109
8	Blood.		116	87		101
	Muscle.		25	45		35
24	Blood.	95	80			88
	Muscle.	42	47			45

Blood sugar was determined by the method of Folin and Wu; muscle sugar by the method of Shaffer and Hartmann on filtrates prepared according to Hoagland.

above the value for a fasting rabbit. At 8 hours the blood sugar has reached nearly normal limits. The muscle sugar, however, has descended to a low level, which continues at the 24th hour. In one of our experiments the muscle sugar was virtually depleted. If low muscle sugar is the cause for insulin-like convulsions, one should expect convulsions after the initial period of adrenalin activity. While our rabbits showed no such symptoms, it is interesting to note that Sahyun and Luck (5) observed

a rabbit, which "for some unknown reason, went into convulsions." This animal described as Rabbit 19 in Table III of their paper was killed 5 hours after receiving 1 mg. of adrenalin. Since these authors used 2 kilo rabbits, their adrenalin dosage was higher than ours.

CONCLUSIONS.

1. The blood sugar and muscle sugar values do not run parallel after the subcutaneous administration of 0.25 mg. of adrenalin per kilo to the rabbit. From the 8th to the 24th hour the muscle sugar is at a very low level. At the 3rd hour the muscle sugar has already fallen to the fasting value, while the blood sugar is at the peak of the curve.

2. The *in vitro* experiments of Sahyun and Alsberg, which indicated that deglycogenated whole skeletal muscle has the power of utilizing glucose *in vitro*, are invalidated by the above observations.

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A STUDY OF GLUTATHIONE.

III. THE STRUCTURE OF GLUTATHIONE.

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Hopkins, in 1929, showed that the copper salt of glutathione could be precipitated in a beautifully crystalline form. He described the isolation of crystalline glutathione and submitted evidence that it was a tripeptide of glutamic acid, glycine, and cysteine (5, 6). It was the discovery of the specific nature of the copper salt that enabled him to separate glutathione in high yield from yeast and blood cells. Hopkins deliberately refrained from committing himself as to the exact structure of glutathione, but he described its chemical properties and pointed out that when this tripeptide was boiled in water, it was decomposed and converted into the anhydride of glycyl cysteine. Pirie and Pinhey (11) determined the physical constants of the two carboxyl, the amino, and the sulfhydryl groups, and suggested two formulas which appeared to be the most probable arrangements of the three amino acids in glutathione.

In recent publications (7–10), Kendall, McKenzie, and Mason have reported the preparation of glutathione in pure crystalline form by a modification of Hopkins' original method. They also showed that the molecule is a tripeptide composed of glutamic acid, glycine, and cysteine, and it was suggested tentatively that the glycine, through its amino group, was attached to the glutamic acid which in turn was attached to the amino group of cysteine. Evidence for this was the fact that hydrogen peroxide in the presence of ammonia destroyed both the glutamic acid and glycine groupings in glutathione. This indicated that the glutamic acid was oxidized at the position of its free amino group and that the subsequent decarboxylation of the glutamic acid resulted in the liberation and ultimate oxidation of the glycine which was attached to this carboxyl group. It was pointed out that this result was negative in character and that until positive results could be obtained, the structure of the tripeptide would remain in doubt.

Additional data have now been obtained; the further work will be reported, although which one of the twelve formulas is the actual structure of glutathione cannot be definitely stated at this time.

Because there are two carboxyl groups in glutamic acid, the number of possibilities for different combinations of the three amino acids is much greater than the number of combinations with 3-monocarboxylic amino acids. Pirie and Pinhey have pointed out that there are twelve different possible arrangements of the three amino and four carboxyl groups. This does not include stereoisomers which depend on spatial configuration.

Goldschmidt, Wilberg, Nagel, and Martin (4) have shown that sodium hypobromite reacts with amino acids and dipeptides. The peptide is broken at the position occupied by the free amino group. This reaction was carried out with glutathione, and it was found that the mononitrile of succinic acid was not liberated in significant amounts but that succinic acid could be separated after subsequent hydrolysis. Glutamic acid could not be recovered following the action of hypobromite, but glycine was recovered in good yield. After the action of alkaline hypobromite, if the solution was made acid and treated with nitrous acid, glycine was not destroyed. These results exclude all possibilities except three. These are: (1) glycyl glutamyl cysteine with the amino group of the glutamic acid alpha to the carboxyl group attached to cysteine, (2) glutamyl cysteinyl glycine with the amino group of the glutamic acid gamma to the attached carboxyl group, and (3) glutamyl glycyl cysteine with the free amino group in the same position as in (2). The formation of glycyl cysteine anhydride by the action of boiling water on glutathione, shown by Hopkins, satisfactorily excludes the first possibility. It can also be excluded by the reaction of glutathione with hypobromite which will be briefly described.

The reaction products given by alkaline hypobromite on compounds with structures (1) and (2) would in each case show the presence of succinic acid after acid hydrolysis but not before. This reaction alone therefore is not conclusive. A more specific reaction is needed; this is furnished by a modification of the treatment with alkaline hypobromite. If the amino group of the glycine is attached to the carboxyl group of the glutamic acid, sodium hypobromite would not liberate more than traces of carbon dioxide from the molecule, unless a deep seated reaction resulted in the oxidation of the cysteine or the glycine or both. The glycine in glutathione is not destroyed by hypobromite.

This excludes all carboxyl groups as the source of carbon dioxide except the carboxyl group of cysteine. This carboxyl group can be excluded by a reaction that will be described later. If glutathione has the second structure, then 1 mol of carbon dioxide would be liberated during the reaction with the hypobromite.

It is thus apparent that if carbon dioxide could be determined in the solution at the time of the oxidation with hypobromite, definite information would be furnished to show whether or not the carboxyl group of the glutamic acid is free. As a preliminary experiment, glutamic acid was dissolved in 2 equivalents of sodium hydroxide, and after the solution had been cooled to 0°, it was added to sodium hypobromite in a 0.1 N sodium hydroxide solution, also cooled to 0°, to which had been added 6 mols of barium chloride. The solution was unclouded with barium carbonate when the sodium salt of glutamic acid was added, but it rapidly became turbid and after 1 or 2 minutes a heavy precipitate of barium carbonate settled from the solution. This was filtered off and dissolved in N hydrochloric acid. 21 cc. were required. 20 cc. would be the theoretical amount for the glutamic acid used. This result indicates that the decarboxylation was complete.

The reaction was repeated with the sodium salt of glutathione which had been oxidized with bromine in acid solution to the sulfonic acid derivative. Barium carbonate formed as with glutamic acid but the time required was slightly longer. At the end of 10 minutes, the barium carbonate was rapidly removed and treated as before. The same amount of hydrochloric acid was required. The alkaline hypobromite had removed 1 mol of carbon dioxide from glutathione. The result would leave no doubt that the carboxyl group of the glutamic acid is unsubstituted, provided the carboxyl group of cysteine could be excluded as the source of the carbon dioxide.

This was excluded by the following reaction: If glutathione had the structure (1), the carboxyl group of the glutamic acid which is attached to the cysteine would be adjacent to the amino group. The product of the reaction of alkaline hypobromite would be a substituted carbamic acid derivative. The work of Goldschmidt and his coworkers has shown that this grouping formed in other di- and tripeptides by hypobromite is relatively stable in alkaline solution but is broken down in acid solution with the liberation of

carbon dioxide. Therefore, if the hypobromite reaction is carried out in the presence of barium hydroxide and the 1 mol of carbon dioxide which is formed is removed, a second mol of carbon dioxide would be rapidly liberated if the solution is made acid.

This experiment was tried with the sulfonic acid derivative of glutathione. Only 8 per cent of 1 mol of carbon dioxide was liberated by acid after treatment with barium hypobromite and removal of the carbon dioxide which had been formed. This reaction excludes the first formula and therefore the carboxyl group of cysteine can be excluded as the source of the carbon dioxide broken off by alkaline hypobromite.

There are three reactions that confirm the position of the free amino group in glutathione. First, if glutathione is oxidized with hydrogen peroxide in the presence of ammonia, succinic acid is not liberated by the oxidation but a significant percentage of the total glutamic acid can be separated as succinic acid after hydrolysis (8, 12). This reaction was first suggested and carried out by Quastel, Stewart, and Tunnicliffe on a sample of glutathione which at that time was described as glutamyl cysteine, but which persumably contained a large percentage of the tripeptide. Second, if glutathione is treated with nitrous acid and then with alkaline sodium hypobromite, succinic acid cannot be isolated until after hydrolysis. Finally, if glutathione is oxidized with chloramine-T, the mononitrile of succinic acid (2) is not liberated in significant amount, but succinic acid can be recovered after hydrolysis. All of these three reactions involve the destruction of the amino group of the glutamic acid and the formation of succinic acid, but one of the carboxyl groups of the succinic acid remains firmly bound until after hydrolysis. The position of the free amino group in glutathione must be gamma to the carboxyl group which attaches the glutamic acid to the rest of the molecule.

Although the reaction products formed by sodium hypobromite, hydrogen peroxide, nitrous acid, and chloramine-T all indicate that the amino group of the glutamic acid is gamma to the carboxyl group which is substituted, one result indicates that the amino group is alpha to the substituted carboxyl group. This is the number of equivalents of sodium hypobromite which are reduced in alkaline solution. Goldschmidt and Strauss (3) have shown that the free amino group in a dipeptide will reduce 4

equivalents of hypobromite with the formation of a nitrile from the amino acid. In tripeptides, however, a second reaction occurs and the first step, which is the formation of the nitrile, is immediately followed by a second reaction; namely, the formation of a hydantoin derivative. In some tripeptides the hydantoin was actually isolated and in others its presence was indicated by the decomposition products. The hydantoin was in each case dehydrogenated by the sodium hypobromite with the reduction of 2 equivalents of hypobromite so that a total of 6 equivalents of hypobromite was reduced by the tripeptide. Glutathione, after the sulfhydryl group had been oxidized in acid solution, reduced 6 equivalents of hypobromite in slightly alkaline solution. This indicates that the amino group is alpha to the carboxyl group which attaches the glutamic acid to the rest of the molecule and that a hydantoin is formed.

The products of the reaction between hypobromite and glutathione show conclusively that a hydantoin derivative cannot be formed. It therefore becomes necessary to determine what part of the molecule is attacked in addition to the free amino group. It seems most probable that the hypobromite in excess of the amount required to react with the amino group of the glutamic acid is reduced at least in part by the formation of a substituted imino group from the substituted amino group of the cysteine. This hypothesis is strengthened by the fact that 2 mols of nitrogen are given off as nitrogen or ammonia when a solution which has been treated with hypobromite is boiled with sodium hydroxide. 1 mol of nitrogen is not liberated as ammonia and it can be shown that this nitrogen is in the form of the amino group of glycine.

If the alkaline hypobromite continues to act for 18 hours, the nitrogen which is not given off as nitrogen gas or as ammonia when the solution is boiled with sodium hydroxide is reduced to a small amount, less than 40 per cent of 1 mol. All of the hypobromite up to 15 equivalents is reduced. The total amount of hypobromite which can be reduced by glutathione depends on the amount of hydrolysis of the nitrile which is formed and the extent to which the C–O–N–H linkages are broken, with the liberation of intermediate products which can further reduce the hypobromite. After the first rapid reaction, during which 1 mol of carbon dioxide is broken off, the liberation of carbon dioxide proceeds slowly, and

at the end of 18 hours about 80 per cent of 1 more mol of carbon dioxide is liberated.

In addition to the formation of carbon dioxide, the solution also contains sulfate ion. Only about 1 per cent of the sulfonic acid was broken off during the first oxidation at 0°. More than 20 per cent of the sulfonic acid was present as sulfate at the end of the oxidation with barium hypobromite after the solution had stood for 18 hours. These results show deep seated destruction of the molecule and indicate that cysteine is the probable source of the carbon dioxide which is broken off in excess of 1 mol, when the alkaline solution of the hypobromite is made acid.

The products which can be extracted with ether from the acidified solution, after oxidation with hypobromite has been carried out for various lengths of time, also indicate the progressive destruction of the tripeptide. Although only from 10 to 15 per cent of 1 mol of the mononitrile of succinic acid can be separated by extraction with ether, after oxidation with hypobromite has been carried out for 5 minutes at 0°, in the ether extract there is present about 10 per cent of 1 mol of some compound containing nitrogen which is not given off as ammonia. This was identified as glycine after the ether extract had been hydrolyzed with hydrochloric acid. Glycine with some substituent attached to the amino group is slowly extracted with ether. After the hypobromite in 0.1 N sodium hydroxide solution had been allowed to act for 18 hours, the mononitrile of succinic acid was not only liberated but the nitrile group was hydrolyzed to the carboxyl group and a large percentage of the total succinic acid formed was extracted with ether although the solution had not been heated above 25° at any time and the alkalinity did not exceed 0.1 N.

Although 6 equivalents of sodium hypobromite are rapidly reduced by glutathione in the form of its sulfonic acid derivative, only 4 equivalents of chloramine-T are reduced. Furthermore, if glutathione is first treated with nitrous acid and the amino group is converted to a hydroxyl group, only 2 equivalents of sodium hypobromite are rapidly reduced and 2 more are slowly reduced if the solution is allowed to stand for 30 minutes at 0°.

The results obtained with nitrous acid, sodium and barium hypobromite, hydrogen peroxide in the presence of ammonia, and chloramine-T have shown that glutathione is either glutamyl

cysteinyl glycine or glutamyl glycyl cysteine, with the amino group of glutamic acid gamma to the carboxyl group which is substituted. These are the same two structures which have already been suggested by Pirie and Pinhey.

If a reaction could show that the carboxyl group of the glycine is free, the possibility that glycine occupies the center position would be excluded. Such a reaction is furnished by the Grignard reagent. Bettzieche, in 1926, suggested this reaction for the determination of the terminal carboxyl group. Phenylmagnesium bromide was added to glutathione ethyl ester and a derivative of diphenylacetaldehyde was isolated. This is evidence that the carboxyl group of the glycine is free; the formula in which glycine is the middle amino acid would be excluded if it could be shown that the tripeptide had not been hydrolyzed during the preparation of the ester.

The ester of glutathione was prepared in absolute alcohol at 0° with hydrochloric acid gas. Whether or not the C—O—N—H linkages of the tripeptide were broken during formation of the ester has not been shown. The hydrolysis of glutathione in water and alcohol is now being further investigated.

Erepsin will split the tripeptide slowly. Under the conditions used practically no reaction occurred within the first 24 to 36 hours, but after digestion for 8 to 10 days, glycine and cysteine could be separated from the solution. The C–O–N–H grouping, therefore, between glutamic acid and the rest of the molecule is not stable to the action of erepsin, although the amino group of the glutamic acid is gamma to the attached carboxyl group. In addition to the effect of erepsin in buffer at pH 7.8 the action of water alone was investigated. Water at 38° will break off glutamic acid in the form of pyrrolidonecarboxylic acid. About 80 per cent of the glutamic acid is liberated after 14 days.

The formula tentatively suggested by us was glutamyl glycine cysteine (7–10). The chief reason for believing that the glycine was attached to glutamic acid was the fact that both the glycyl and glutamyl groupings in glutathione are destroyed by the action of hydrogen peroxide at 70° on the ammonium salt of glutathione. The results reported in this paper show that the destruction of the glycyl grouping with hydrogen peroxide cannot be used as a reaction to determine the position of the glycine.

Hopkins (6) has made available a method for the isolation of glutathione which can be applied for the quantitative removal of this tripeptide as its copper salt. A comparison of certain details of the method as published by Hopkins, however, with the method suggested by us (8) made it seem probable that a combination of the two methods would furnish the maximal yield with a minimal amount of time and material.

Suspension of yeast in cold water and benzene removes all the glutathione which can be removed by heating the solution and if a large centrifuge is available it simplifies the early stages of the isolation of glutathione. Furthermore, precipitation of glutathione with lead acetate at a pH of 5.5 is practically quantitative. There is no advantage in adding mercury sulfate and there is a distinct disadvantage in raising the pH to 8 or 9 as very little glutathione, not more than 1.5 gm. from 45 kilos, is precipitated, but the higher pH causes the lead acetate to carry down a large amount of interfering substances. The lead precipitate is decomposed with sulfuric acid and after the solution has been adjusted to the proper acidity, it is ready for the precipitation with copper.

Hopkins (6) found that if the copper is removed from the solution after precipitation of glutathione with cuprous oxide and the glutathione not precipitated with copper is removed with mercury sulfate, retreatment of a solution of the mercury precipitate yields about 20 per cent as much glutathione as is obtained in the first precipitate with copper. If glutathione is separated with the cold water-benzene technique, it is practically quantitatively precipitated with copper at the first treatment. Almost no glutathione can be recovered with mercury sulfate from the solution filtered from the copper precipitate.

The yield of glutathione in gm. from eight 45 kilo lots of yeast was: 4.00, 4.95, 4.1, 16.1, 3.6, 14.9, 10.7, and 20.7. Separation of the glutathione through crystallization as we have previously described was the only method used to isolate the tripeptide. For the last fifteen lots, the following yields were obtained: 21.0, 39.5, 31.0, 34.4, 33.7, 31.0, 33.7, 21.5, 18.9, 17.0, 29.0, 29.8, 31.4, 30.3, 31.6. For these, a combination of Hopkins' method and our method which is outlined in the description of the experimental data was used.

In the first paper on the isolation of glutathione, it was pointed

out that the yields were variable and it was suggested that possibly enzyme action caused the breakdown of some of the tripeptide. This appeared probable because of the high sulfur content of preparations of glutathione which have been reported. Several preparations of glutathione contained as high as 12.33 per cent of sulfur. We, therefore, assumed that one of the amino acids was probably broken off either in the yeast cell or in the solution after the glutathione had been removed from the yeast with benzene.

Up to the present time, no explanation has been suggested for the high sulfur content which was found in the samples of glutathione prepared by several investigators. The liberation of glutamic acid with boiling water reported by Hopkins and the slow progress of this reaction even at 38° reported in this paper, indicate that a dipeptide of cysteine and glycine may be one of the compounds that explains the high content of sulfur in some samples of glutathione.

Experiments were carried out to determine the loss of glutathione in solutions which had been allowed to stand. When this was first tried, the solution of the yeast after treatment with benzene was divided into two equal portions. One portion was worked up as usual without delay and the other portion was allowed to stand for about 24 hours. The yield of glutathione in the solution which had stood was much reduced. Crystallization of the glutathione was the only method employed for its separation. When this same experiment was repeated with precipitation by cuprous oxide it was found that the yield of glutathione was not less in the solution which had stood. These results suggest that there are substances which interfere with the crystallization of glutathione and that it is failure to crystallize the tripeptide rather than a destruction of the substance which explains the variable and low yields in some lots of yeast. 20.7 gm. of crystalline glutathione were separated in one lot without the use of copper precipitation and yet in another the yield was only 3.6 gm. We cannot give an explanation for this variation in yield at this time, but we have found that some of the earlier samples of glutathione which were prepared by precipitation with alcohol did not give a precipitation with cuprous oxide. As much as 30 gm. of the material when dissolved in sulfuric acid and treated with small amounts of cuprous oxide failed to give more than 0.5 gm. of the copper precipitate.

This material, if dissolved in water, 50 per cent alcohol, or dilute acetic acid, would not crystallize. However, it possessed almost the theoretical content of the sulfhydryl group as shown by the electrometric titration with potassium ferricyanide. Its sulfur content agreed with that of the tripeptide and its nitrogen was only slightly below the theoretical amount. It is improbable that the failure to crystallize or precipitate with copper is due to oxidation to the -SS form because precipitation with mercury and treatment with hydrogen sulfide presumably completely reduce the sulfur to the sulfhydryl form; but this does not induce its crystallization. The material was treated with phosphotungstic acid at 0°, but this did not remove impurities and permit crystallization.

In the filtrate of the lead acetate precipitation from yeast, some sulfhydryl compound is still present which is not glutathione. The mercury precipitate of this sulfur compound is more difficultly soluble in acid than is the mercury precipitate of glutathione. Its isolation in crystalline form is difficult because of the other substances which have practically the same solubilities in the presence of heavy metals and phosphotungstic acid.

EXPERIMENTAL.

Action of Nitrous Acid on Glutathione.—The results obtained by treatment of glutathione with nitrous acid have been published (8, 12).

Evidence That the Carboxyl Group of Glycine Is Free (1).—3 gm. of crystalline glutathione were suspended in 25 cc. of absolute alcohol and saturated with hydrochloric acid gas at 0°. The solution was evaporated to dryness under reduced pressure, dissolved in 25 cc. of absolute alcohol, and again saturated at 0° with hydrochloric acid gas. Evaporation and saturation were repeated a third time. The solution then stood 18 hours in the ice box. The alcohol and hydrochloric acid were removed under reduced pressure and the last traces were removed with an efficient vacuum pump. The porous brittle mass was easily removed from the flask. It was not shown to be a pure preparation of the diester hydrochloride, but since it reacted with phenylmagnesium bromide, as described, some of the carboxyl group of the glycine must have been present in the form of the ethyl ester. The material was powdered and kept in a desiccator until used. It is quite

hydroscopic. The Grignard reagent was prepared from 6 gm. of magnesium and 44 gm. of phenylbromide added to 150 cc. of anhydrous alcohol-free ether. After the reaction was complete, the dry powdered ester hydrochloride was added in small portions with shaking. The solution was heated under a reflux condenser for 2 hours. The reaction mixture was decomposed with ice-cold hydrochloric acid, 1:1. A gummy material precipitated and stuck to the flask. It was washed with ether and dissolved in a small amount of hot alcohol containing hydrochloric acid. The hydrochloride of the peptide alcohol was precipitated with ether as a slightly brownish powder. It is quite insoluble in water and ether. The free base is soluble in ether and insoluble in water and alcohol. Yields of the peptide hydrochloride varied from 40 to 62 per cent. Identity of the product was not established beyond the fact that its chemical properties were similar to the products obtained by Bettzieche. The peptide alcohol was hydrolyzed under varying conditions. The best yield was obtained as follows: 0.85 gm. of the peptide alcohol was dissolved in 125 cc. of alcohol containing 100 cc. of concentrated hydrochloric acid. 55 cc. of water were added and the solution was refluxed for 5.5 hours. 100 cc. of water were added and the mixture distilled. Water was added from time to time until the distillate was 400 cc. This was extracted with ether five times. The ether was dried with sodium sulfate and distilled; to the residue was added 0.5 gm. of hydrazine sulfate in 50 cc. of water. The solution was shaken thoroughly. The solid material was scraped from the flask and crystallized from alcohol. The yield was 0.036 gm. This is 13 per cent of theory. The product melted from 169–171°. The material mixed with a sample of diphenylacetaldazine had the same melting point. Three separate preparations of the ester yielded the same final product. Bettzieche has carried out a large amount of work on this compound in connection with the determination of the position of glycine in peptides. The method of formation and the physical and chemical properties of the azine obtained from glutathione were identical with those of the azine of diphenylacetaldehyde. Particular care was taken in one preparation of the ester to keep the temperature low: it did not rise above 40° at any time. The low yield of the azine may be explained by the reactivity of the diphenylacetaldehyde. A long hydrolysis is necessary to

break up the peptide alcohol. During this treatment, the aldehyde may enter into side reactions, particularly with the -SH group. Bettzieche has shown that diphenylacetaldehyde is converted into desoxybenzoin by heating with strong acid. The azine was prepared from glycine ethyl ester hydrochloride but the yield in this case was also low, 33 per cent of theory.

Glycine could not be separated as hippuric acid after the peptide alcohol had been hydrolyzed with hydrochloric acid. This fact further indicates that glycine is not in the middle position in the tripeptide.

Oxidation of Glutathione to Its Sulfonic Acid Derivative.—In all of the experiments with solutions of sodium or barium hypobromite, the sulfonic acid derivative of glutathione was prepared before the solution was added to the hypobromite. The sulfhydryl group of 0.01 mol of the tripeptide was oxidized to the sulfonic acid by the addition of bromine to 50 cc. of cold N hydrochloric acid in which the glutathione was dissolved. In some experiments, 0.04 mol of glutathione in 200 cc. of N hydrochloric acid was oxidized at 0°. The solution was then divided into four portions. The amount of bromine required for the oxidation was found to be close to the theoretical in each case. If more bromine was added after the end-point was reached, it was slowly reduced. This could be continued up to about 10 per cent of the total bromine added. The sulfonic acid was not separated in crystalline form but the reactions with sodium hypobromite clearly show that oxidation of the sulfhydryl group did not cause hydrolysis of the tripeptide.

Action of Sodium Hypobromite; Number of Equivalents Reduced.—0.01 mol of the sulfonic acid derivative of glutathione in 50 cc. of N hydrochloric acid was neutralized with 28 cc. of 5 N sodium hydroxide. The total volume of the solution was 80 cc.; it was cooled to 0° and added to a solution containing 400 cc. of 0.385 N sodium hypobromite and 10 cc. of 5 N sodium hydroxide, cooled to 0°. The total volume of the solution was 490 cc. The alkalinity was 0.1 N. The reduction of the hypobromite expressed in terms of 0.1 N was as follows: 30 minutes, 617 cc.; 44 minutes, 677 cc.; 60 minutes, 732 cc.; 77 minutes, 977 cc. With the same amount of the sulfonic acid derivative and hypobromite in the presence of 0.1 N barium hydroxide, the following amounts of hypobromite

were reduced. After 20 minutes the solution was allowed to stand at room temperature; 8 minutes, 532 cc.; 21 minutes, 612 cc.; 70 minutes, 822 cc.; 18 hours, 1500 cc. A neutral solution of 0.01 mol of the sulfonic acid derivative of glutathione was added to 1556 cc. of 0.1 N sodium hypobromite at 0°. The total volume was 600 cc. The solution contained 0.1 N sodium hydroxide. 564 cc. of 0.1 N hypobromite were reduced. In a similar experiment the order of addition was reversed; the same amount of hypobromite was added slowly to the sodium salt of the sulfonic acid derivative of glutathione. The volume, temperature, and concentration of sodium hydroxide were the same. The same amount of hypobromite was reduced.

12 gm. of glutathione, oxidized to its sulfonic acid derivative, were treated with 800 cc. of 0.4 N sodium hypobromite in 0.1 N sodium hydroxide solution. 2194 cc. of 0.1 N hypobromite were reduced at the end of 25 minutes. This is 5.64 equivalents of hypobromite for each mol of glutathione.

Effect of Hypobromite on the Nitrogen in Glutathione.—0.039 mol of the sulfonic acid derivative of glutathione in a neutral solution was added to 3147 cc. of 0.1 N sodium hypobromite. There were present in the glutathione 1172 cc. of 0.1 N nitrogen. 164 cc. of 0.1 N nitrogen were given off as a gas. 1008 cc. of 0.1 N nitrogen remained in the solution. The equivalent of 636 cc. of 0.1 N ammonia was liberated when an aliquot part of the solution was boiled with sodium hydroxide. 372 cc. of 0.1 N nitrogen (95.6 per cent of 1 mol) were stable to sodium hydroxide. 800 cc. of 0.1 N nitrogen (2 mols) were liberated as nitrogen or as ammonia.

In the presence of barium hydroxide, sodium hypobromite at 0° may break off almost no nitrogen. 291 cc. of 0.1 N nitrogen (97 per cent) were present after 0.01 mol of glutathione had been treated with excess of sodium hypobromite. Of this amount 164 cc. were liberated as ammonia with sodium hydroxide. In another similar experiment, 251 cc. of 0.1 N nitrogen (84 per cent) were present after the treatment with sodium hypobromite. Of this, 130 cc. were liberated as ammonia. 121 cc. of 0.1 N nitrogen were not liberated as nitrogen or ammonia.

0.01 mol of the sulfonic acid derivative of glutathione was allowed to stand in the solution of barium hypobromite for 18 hours. There were present in the solution 150 cc. of 0.1 N nitro-

gen. Of this amount 123 cc. were given off as ammonia. Only 37 cc. of 0.1 N nitrogen were stable to sodium hydroxide after the prolonged action of hypobromite.

Action of Nitrous Acid Followed by Sodium Hypobromite; Stability of Nitrogen.—0.01 mol of the sulfonic acid derivative of glutathione was prepared and the ammonia which was liberated with strong sodium hydroxide was determined on an aliquot portion. 34 cc. of 0.1 N ammonia were liberated from the 0.01 mol. After treatment with nitrous acid 0.01 mol of the sulfonic acid derivative of glutathione liberated 20 cc. of 0.1 N ammonia. After the same solution had been treated with sodium hypobromite following the action of the nitrous acid 61 cc. of 0.1 N ammonia were liberated by sodium hydroxide. This indicates that the substituted amino group of the cysteine is attacked by the hypobromite since the ammonia liberated after the action of nitrous acid must be derived from the amino group of cysteine.

Action of Sodium Hypobromite on Glutamic Acid.—1.46 gm. of glutamic acid were dissolved in 20 cc. of N sodium hydroxide and cooled to 0°. This was added to 400 cc. of 0.4 N sodium hypobromite in 0.1 N sodium hydroxide. 420 cc. of 0.1 N hypobromite were reduced. The excess hypobromite was destroyed with 30 per cent hydrogen peroxide. 12 cc. of 5 N hydrochloric acid were added. The solution which contained 87 cc. of 0.1 N nitrogen was concentrated to 60 cc. and extracted with ether in a continuous extractor. After extraction the solution contained 5 cc. of 0.1 N nitrogen. It was assumed that the only form of nitrogen soluble in ether would be the mononitrile (2) of succinic acid or products formed from the nitrile. It is possible that partial hydrolysis or rearrangement of the nitrile occurs since a small amount of a nitrogen-containing substance with a much higher melting point than the nitrile was present in the ether solution. Identity of this product was not established but it was always found in the ether extract of glutamic acid after treatment with sodium hypobromite. The ether was evaporated from the extracted material and in some experiments the nitrile was separated in crystalline form. When the products soluble in ether were boiled in barium hydroxide, all of the nitrogen was given off as ammonia. The precipitate of barium succinate was well washed with hot water and decomposed with the exact amount required of sulfuric acid. Yield 700 mg.

of succinic acid. This is 60 per cent of the theoretical amount. It was recrystallized from water; m.p. 188°. 100 mg. neutralized 16.8 cc. of 0.1 N sodium hydroxide.

Action of Sodium Hypobromite. Evidence As to the Possible Formation of the Mononitrile of Succinic Acid from Glutathione (*2*).—0.01 mol of the sulfonic acid derivative of glutathione in neutral solution at 0° was added to 400 cc. of 0.4 N hypobromite in 0.1 N sodium hydroxide at 0°. The excess hypobromite was destroyed with 30 per cent hydrogen peroxide and the solution was made acid with 20 cc. of 5 N hydrochloric acid. After removal of the last traces of bromine with a water suction pump, the solution was concentrated to 60 cc. and extracted with ether for 7 hours; the ether was evaporated and the residue dissolved in water to which barium hydroxide had been added. The solution was boiled and the ammonia which was given off was caught in sulfuric acid. 11 cc. of 0.1 N sulfuric acid were neutralized. The barium precipitate was filtered from the boiling hot solution and was just decomposed with sulfuric acid. 9 mg. of succinic acid were formed from the barium succinate. This experiment was repeated several times. The maximal amount of the nitrile extracted with ether estimated by the nitrogen content was less than 20 per cent of 1 mol. In some experiments it was less than 6 per cent. The actual isolation of the nitrile in crystalline form was impossible because so little of this compound was mixed with a large amount of interfering substances. Although the pure nitrile was not isolated from glutathione, the results show conclusively that it was not liberated in significant amount.

Action of Sodium Hypobromite; Separation of Succinic Acid.—The solution which had been extracted with ether was hydrolyzed with constant boiling hydrochloric acid for 8 hours. It was evaporated to dryness and the residue extracted with ether. The ether extract was dissolved in water and the succinic acid precipitated from the boiling hot solution as its barium salt. This was filtered from the solution and decomposed with sulfuric acid. 600 mg. of succinic acid were obtained. This is 51 per cent of the theoretical amount. It was recrystallized from water; m.p. 188°. 100 mg. neutralized 16.8 cc. of 0.1 N sodium hydroxide. This experiment was repeated several times. If the concentration of the solution after the action of sodium hypobromite was not carried

out at a low temperature, succinic acid was frequently found in the first ether extract. If, however, the solution was concentrated at a temperature below 25° and the amount of hydrochloric acid was only slightly in excess of the amount sufficient to liberate the carboxyl and sulfonic acid groups, only traces of succinic acid or the nitrile were found in the first ether extract. In a similar experiment with 0.1 N barium hydroxide and 1500 cc. of sodium hypobromite which stood at room temperature for 18 hours, 381 mg. of succinic acid (32 per cent) were recovered from the first ether extract after precipitation as barium succinate.

Action of Sodium Hypobromite, Destruction of Glutamic Acid, and the Recovery of Glycine.—0.01 mol of the sulfonic acid derivative of glutathione was treated with sodium hypobromite for 4 minutes at 0°. It was then hydrolyzed with constant boiling hydrochloric acid for 6 hours, concentrated to a small volume, and saturated with hydrochloric acid gas. The sodium chloride was filtered off and the solution was further concentrated and saturated with hydrochloric acid. A small amount of ammonium chloride separated but glutamic acid hydrochloride was not present. The solution after extraction with ether was made 0.5 N with sodium hydroxide in a volume of 150 cc. and treated with 5 cc. of benzoyl chloride. It was shaken frequently for a period of 4 hours, acidified with hydrochloric acid, and extracted six times with ethyl acetate (200 cc. total volume). The ethyl acetate was completely removed and the residue dissolved in chloroform. After standing for 24 hours, 0.976 gm. of hippuric acid separated. This is a yield of 54 per cent of the glycine. 0.01 mol of the sulfonic acid derivative that had stood for 18 hours at 25° and had reduced 15 equivalents of hypobromite was hydrolyzed and benzoylated. Only 374 mg. of hippuric acid were recovered.

Action of Sodium Hypobromite Followed by Treatment with Nitrous Acid. Recovery of Glycine.—0.01 mol of the sulfonic acid derivative of glutathione was treated with sodium hypobromite as described. The excess hypobromite was destroyed with 30 per cent hydrogen peroxide and the solution was made acid with 20 cc. of 5 N hydrochloric acid. 2.1 gm. of sodium nitrite were added; the solution was concentrated to 60 cc., and extracted with ether. Concentrated hydrochloric acid was then added until a solution of constant boiling acid was present and the solution was

boiled for 6 hours. It was neutralized and made 0.5 N with sodium hydroxide. The solution was benzoylated as described; 1.045 gm. of hippuric acid were recovered. This is a yield of 60 per cent of the theoretical.

Action of Sodium Hypobromite on Glutamic Acid, and Liberation of Carbon Dioxide.—0.01 mol of glutamic acid (1.46 gm.) was dissolved in 20 cc. of N NaOH. 200 cc. of 0.4 N sodium hypobromite were cooled to 0° and 160 cc. of water containing 15 gm. of barium chloride and 40 cc. of N sodium hydroxide were added to the hypobromite. The solution of glutamic acid was diluted to 40 cc. and cooled to 0°. After 5 minutes, the solution was filtered with suction and the precipitate was well washed. It was suspended in distilled water to which N hydrochloric acid was added. 21 cc. were required to dissolve the barium carbonate. The solution was filtered and the barium precipitated as sulfate. The weight of the barium sulfate was 2.4482 gm. This is 0.0105 mol.

Formation of Carbon Dioxide from Glutathione with Sodium Hypobromite.—0.01 mol of the sulfonic acid derivative of glutathione in 50 cc. of N hydrochloric acid was neutralized with 28 cc. of 5 N sodium hydroxide. The solution was diluted to 100 cc. and cooled to 0°. 200 cc. of 0.385 N sodium hypobromite, 40 cc. of N sodium hydroxide, and 60 cc. of water containing 10 gm. of barium chloride were placed in a 500 cc. flask and cooled to 0°. The glutathione solution was then slowly added to the hypobromite. At the end of 10 minutes, the solution was rapidly filtered through a Buchner filter, and the barium carbonate was well washed with water and dissolved with N hydrochloric acid. 21 cc. were required. The solution was heated to boiling until all traces of carbonate had been dissolved. A slight excess of hydrochloric acid was added and the solution filtered. The barium precipitated as sulfate weighed 2.399 gm. This is 0.0103 mol. A similar experiment carried out with 1500 cc. of 0.1 N hypobromite gave 0.0104 mol of carbon dioxide after 4 minutes determined as barium sulfate and 0.0086 mol more after 18 hours.

Carbon Dioxide Liberated by Acid after Alkaline Hypobromite.—0.01 mol of the sulfonic acid derivative of glutathione in 50 cc. of N hydrochloric acid was neutralized with 28 cc. of 5 N sodium hydroxide. The solution was diluted to 100 cc. 150 cc. of 0.4 N sodium hypobromite, 30 cc. of N sodium hydroxide, and 20 cc. of

water with 10 gm. of barium chloride were placed in a flask and cooled to 0°. The solution of glutathione was added slowly to the hypobromite. After 10 minutes the solution was filtered. It was then acidified with 20 cc. of 5 N hydrochloric acid and carbon dioxide-free air was passed through the solution and then through a solution of 200 cc. of 0.11 N barium hydroxide. After 45 minutes, the solution of barium hydroxide free from barium carbonate was titrated. 15 cc. of 0.10 N barium hydroxide had been neutralized. The air was passed through 200 cc. more of 0.11 N barium hydroxide for 3 hours. No more carbon dioxide was liberated during this time. The barium carbonate was dissolved in hydrochloric acid and precipitated as sulfate. It weighed 0.2028 gm. This is 0.0008 mol.

Action of Sodium Hypobromite. Nitrogen Compounds Extracted with Ether.—It was observed that in all of the ether extracts of solutions which were made acid after the action of sodium hypobromite, there was a small amount of material difficultly soluble in the ether. This material was not succinic acid. Ether extracts of several experiments in which glutathione had been treated with sodium hypobromite were analyzed for total nitrogen and for nitrogen liberated as ammonia. In one experiment with 0.01 mol of the sulfonic acid derivative of glutathione which had been treated with sodium hypobromite for 4 minutes there was a total of 25 cc. of 0.1 N nitrogen. Sodium hydroxide liberated 15 cc. of 0.1 N ammonia. In a second similar experiment there was present a total of 30 cc. of 0.1 N nitrogen. Sodium hydroxide liberated 19 cc. of 0.1 N ammonia. In a third experiment there was present a total of 21 cc. of 0.1 N nitrogen. Sodium hydroxide liberated 15 cc. of 0.1 N ammonia. The three ether extracts were hydrolyzed with hydrochloric acid and were treated with benzoyl chloride. 34 mg. of hippuric acid were separated from the first, 62 mg. from the second, and none from the third.

Liberation of Sulfuric Acid from the Sulfonic Acid Derivative of Glutathione.—0.01 mol of the sulfonic acid derivative of glutathione was neutralized and added to a solution containing 600 cc. of 0.1 N sodium hypobromite in 0.1 N sodium hydroxide which also contained 10 gm. of barium chloride. The barium carbonate which formed during the first 4 minutes was removed by filtration. This was dissolved in hydrochloric acid. The barium sulfate

which was formed during the first 4 minutes was 0.00011 M. This is 1 per cent of the amount present. After the solution had stood at room temperature for 18 hours it was again filtered. The barium sulfate present was 0.00214 mol; 21 per cent of the sulfonic acid present.

Determination of the Position of the Free Amino Group in Glutathione. Hydrogen Peroxide in the Presence of Ammonia.—Succinic acid can be isolated after glutathione has been oxidized with hydrogen peroxide in the presence of ammonia only after the solution has been hydrolyzed with acid. These results have been published (8, 12).

Action of Nitrous Acid Followed by Sodium Hypobromite; Isolation of Succinic Acid.—0.01 mol of the sulfonic acid derivative of glutathione in 50 cc. of N hydrochloric acid was treated with 1.5 gm. of sodium nitrite. The solution was neutralized with 21 cc. of 5 N sodium hydroxide and diluted to 90 cc. This was added to a solution containing 600 cc. of 0.1 N sodium hypobromite at 0°. After 12 minutes 320 cc. of hypobromite were reduced. The solution was made acid with 12 cc. of 5 N hydrochloric acid and was concentrated to 50 cc. in a vacuum and extracted with ether. The ether contained 73 cc. of organic acid. The ether was removed and the succinic acid precipitated with barium hydroxide from a boiling solution. The barium succinate was decomposed with sulfuric acid. The succinic acid weighed 30 mg. The solution which had been extracted with ether was then hydrolyzed with hydrochloric acid, evaporated to dryness in a vacuum, and the residue extracted with ether. Succinic acid was precipitated from an aqueous solution of the ether extract with barium hydroxide. The barium succinate was decomposed with sulfuric acid. The succinic acid weighed 131 mg.

Action of Chloramine-T.—0.01 mol of the sulfonic acid derivative of glutathione in neutral solution was treated at 25° with 6 gm. of chloramine-T which was added in the solid condition. This amount of the oxidizing agent liberated 400 cc. of 0.1 N iodine. After 1 hour the glutathione had reduced all except 63 cc. of 0.1 N chloramine-T. The solution was treated with a small amount of sodium bisulfite, concentrated to 60 cc., and extracted with ether to remove the sulfonamide. The solution was then made acid with 15 cc. of 5 N hydrochloric acid and was again ex-

tracted with ether. The ether extract was evaporated to dryness, a solution of barium hydroxide was added, and the succinic acid was precipitated as barium succinate. The barium precipitate was decomposed with just sufficient sulfuric acid and the solution was evaporated to dryness. The residue weighed 8 mg. The solution which had been extracted with ether was hydrolyzed with hydrochloric acid and evaporated to dryness. The dry residue was extracted with ether and treated with a solution of barium hydroxide. The barium precipitate was decomposed with sulfuric acid and the barium sulfate was removed. The filtrate was evaporated to dryness. The residue weighed 23 mg. The same experiment was repeated with 0.01 mol of the sulfonic acid derivative of glutathione and 9 gm. of chloramine-T which liberated 600 cc. of 0.1 N iodine. After 40 minutes 412 cc. of 0.1 N chloramine-T had been reduced. The solution was extracted with ether, then made acid, and reextracted with ether. The second ether extract was concentrated to a small volume to which barium hydroxide was added. 6.3 cc. of 0.1 N ammonia were liberated. The barium succinate was filtered off and decomposed with sulfuric acid. The residue obtained by evaporating the filtrate after the removal of the barium sulfate weighed 24 mg. The solution of glutathione which had been treated with chloramine-T and extracted with ether was then hydrolyzed with hydrochloric acid and reextracted with ether. The succinic acid in the ether solution was precipitated as its barium salt. After this was decomposed, with sulfuric acid, 118 mg. of succinic acid were recovered.

Hydrolysis of the Tripeptide with Erepsin.—Erepsin was prepared from the mucosa of rabbits intestines by extraction of 1 part of the scraped mucosa with 2 parts of 87 per cent glycerol for 7 hours. The glycerol extract was centrifuged. 3 gm. of crystalline glutathione were dissolved in 15 cc. of the glycerol extract. To this were added 35 cc. of phosphate buffer, pH 7.7, and 10 cc. of N sodium hydroxide. The resulting pH was 7.8. The solution was allowed to digest at 37° under toluene and in the presence of a small amount of thymol for various lengths of time, from 14 hours to 15 days. The solution was then acidified with 1 cc. of 5 N sulfuric acid, filtered, and treated with mercury sulfate. In the experiments that stood from 14 to 38 hours, the glutathione was

only slightly hydrolyzed. The solution obtained from the mercury precipitate with hydrogen sulfide gave a heavy precipitate with cuprous oxide. After standing from 8 to 15 days, a precipitate with cuprous oxide could not be obtained from similar solutions.

Cystine.—For the isolation of cystine the mercury precipitate was decomposed with hydrogen sulfide, the solution was filtered, and the hydrogen sulfide removed with air. Sulfuric acid was removed exactly with barium hydroxide and the solution was evaporated to a small volume. 1 drop of ammonia was added and the solution was aerated until the nitroprusside reaction was negative. After the solution had stood for several hours, it was filtered. The yield of cystine obtained was as follows: after 66 hours, 0.300 gm.; 12 days, 0.420 gm.; 13 days, 0.425 gm.; 14 days, 0.450 gm.; 15 days, 0.680 gm. 0.680 gm. is 58 per cent of the theoretical amount of cystine. The purity of the cystine was determined by the color produced by it compared with the color produced by a known sample of cystine when both were treated with the Folin reagent for cystine. It was found to be identical to a sample of pure cystine. Nitrogen by Kjeldahl analysis, 11.4 per cent. Theoretical, 11.67 per cent.

Glycine.—The glycine in the solution after hydrolysis with erepsin was recovered from the mercury filtrate of the cystine. The mercury was removed with hydrogen sulfide; the hydrogen sulfide was removed by aeration. The solution was evaporated to a small volume and was benzoylated in the presence of 0.5 N sodium hydroxide and benzoyl chloride. The following yields of hippuric acid, m.p. 188°, were obtained: after 66 hours, not determined; 8 days, 0.560 gm.; 12 days, 0.325 gm.; 13 days, 0.720 gm.; 14 days, 0.420 gm.; 15 days, 0.710 gm. This is a yield of 40 per cent of the theoretical amount.

Glutamic Acid.—Attempts to isolate glutamic acid from the mercury filtrate after the removal of cystine were as follows: The sulfates and phosphates were removed by addition of barium hydroxide, the solution was filtered, and the barium was then removed with excess sulfuric acid. It was evaporated to 20 cc., 4 cc. of 5 N sulfuric acid were added, and the solution made to 80 per cent in alcohol. The alkaline sulfates formed from the sodium and potassium in the buffer were removed. The alcohol was evaporated and the sulfuric acid was exactly removed with barium

hydroxide. The solution was then evaporated to a small volume and 300 cc. of absolute alcohol and 200 cc. of ether were added. The precipitate which should contain the glycine and glutamic acid was dissolved in 2 cc. of water and saturated with hydrochloric acid gas at 0°. Glutamic acid hydrochloride could not be separated. This solution, however, yielded hippuric acid after benzoylation. It is probable that the glycerol interfered with the separation of the glutamic acid. Further work is in progress.

Distribution of Nitrogen.—After the failure to isolate glutamic acid from the solution obtained by digestion of glutathione with erepsin, the distribution of nitrogen was determined. After the 1 cc. of 5 N sulfuric acid had been added and the coagulated proteins from the enzyme were removed by filtration, 96.3 per cent of the total nitrogen in the glutathione was present in the solution. The filtrate from the mercury precipitate contained 55 per cent of the total nitrogen. This is 82.5 per cent of the 2 mols of nitrogen which should be present as glycine and glutamic acid. The mercury precipitate contained 135 per cent of the nitrogen which should be present in the cystine. This is further evidence confirming the presence of some incompletely hydrolyzed tripeptide in the mercury precipitate even after digestion for 15 days.

Hydrolysis of Glutathione in Water.—3 gm. of crystalline glutathione dissolved in 50 cc. of distilled water were covered with toluene and a small amount of thymol and allowed to stand for 14 days at 38°. At the end of this time, Sullivan's test (13, 14) for cysteine was negative. The solution was precipitated with the mercury sulfate reagent and filtered. 86 per cent of 1 mol of nitrogen was not precipitated with mercury. Mercury and sulfates were removed from the filtrate which was evaporated to 5 cc. in a vacuum at a temperature less than 40°. It was further concentrated and allowed to stand for 18 hours. A mass of crystals separated. They were suspended in ether, filtered, and dried. 550 mg. of this material were extracted with alcohol. The residue insoluble in alcohol weighed 95 mg. From the alcoholic extract 370 mg. of pyrrolidonecarboxylic acid were separated; m.p. 162°. When mixed with a sample of pyrrolidonecarboxylic acid, the melting point did not change. Nitrogen content 0.1182 gm. contained 0.0126 gm. of nitrogen by Kjeldahl. Found, 10.66 per cent. Theoretical, 10.85 per cent. 0.0212 gm. required 1.62 cc.

of 0.1 N sodium hydroxide. This indicates the combining weight of 131. Theoretical, 129.

Method for the Preparation of Glutathione.—45 kilos of bakers' yeast are divided equally among four 75 liter crocks. 36 liters of distilled water and 2400 cc. of benzene are added to each crock and the mixture is thoroughly stirred with a mechanical stirrer. The solutions are allowed to stand for 4 hours at room temperature, 25°. 10 liters of water are added to each crock. 800 cc. of concentrated sulfuric acid are added to 12 liters of water and the solution is cooled. One-fourth of the acid is then poured into each crock. This is followed immediately with an equivalent amount of barium hydroxide, about 1200 gm., for each crock, dissolved in 3 liters of hot water. The formation of barium sulfate in the solution is a great aid when the cells are removed.

The solution is passed through a Sharples supercentrifuge, and is again divided into four equal parts. From 300 to 500 gm. of neutral lead acetate are added to each crock. The pH of the solution after addition of the lead acetate is about 5.5. The lead precipitate is removed in the centrifuge and is decomposed in 5 liters of water to which a slight excess of sulfuric acid is added. About 900 cc. of 5 N acid are usually necessary. The solution is stirred mechanically with a nickel stirrer.

The lead sulfate is separated in the centrifuge and the clear solution is made 0.5 N with sulfuric acid. Congo red is used as indicator. The solution is warmed to 50° and is treated with small portions of cuprous oxide as prepared and used by Hopkins. About 10 gm. are required. The end-point is determined by the failure to produce a precipitate with cuprous oxide in a small part of the solution which has been clarified by centrifugation. The addition of excess cuprous oxide must be avoided.

The solution containing the copper precipitate is allowed to settle for from 3 to 18 hours. The solution is filtered on a pad of infusorial earth. The precipitate is washed and is then suspended in water and decomposed with hydrogen sulfide.

The copper sulfide is filtered out and the sulfuric acid exactly removed with barium hydroxide. The barium sulfate is removed and the solution is concentrated in a high vacuum to a thick syrup. The solution is allowed to stand for 2 days in the ice box, until crystallization is complete. The crystals are filtered and washed

with small portions of glacial acetic acid, and then with absolute alcohol. The glutathione is dried in a vacuum desiccator over sulfuric acid. A second crop of from 2 to 4 gm. can be secured by concentration of the mother liquor of the first crystals. Glutathione cannot be recovered from the acetic acid and alcohol washings.

SUMMARY.

The ethyl ester hydrochloride of glutathione was treated with phenylmagnesium bromide. The peptide alcohol formed was hydrolyzed and diphenylacetaldehyde was obtained as its azine. Unless the tripeptide was hydrolyzed during the preparation of the ester, the separation of the azine indicates that the carboxyl group of glycine is free, and that the glutamic acid is attached to cysteine.

Nitrous acid and sodium hypobromite in alkaline solution destroyed the glutamyl grouping of glutathione, but the glycine could be recovered after hydrolysis.

1 mol of carbon dioxide was broken off at the time the glutamic acid was converted into the mononitrile of succinic acid by alkaline hypobromite, but only traces of carbon dioxide in excess of 1 mol were given off when such a solution was made acid.

The mononitrile of succinic acid formed by hypobromite was not liberated by the action of the hypobromite but succinic acid was separated after hydrolysis with acid.

Nitrous acid did not destroy the glycyl grouping of glutathione after a solution of the tripeptide had been treated with sodium hypobromite.

Chloramine-T oxidized the glutamyl grouping of glutathione to the mononitrile of succinic acid but the nitrile was not broken off from the rest of the molecule until after hydrolysis with acid.

Bromine in acid solution oxidized glutathione to a sulfonic acid. Sodium hypobromite at room temperature liberated about 20 per cent of the sulfur as sulfuric acid from the sulfonic acid derivative of glutathione.

The data obtained show that glutathione is either glutamyl cysteinyl glycine or glutamyl glycyl cysteine. The amino group of the glutamic acid is gamma to the carboxyl group which is substituted.

Erepsin will hydrolyze glutathione into its three amino acids.

Glutamic acid is liberated from the tripeptide in a sterile water solution at 38°.

An improved method for the isolation of glutathione is given.

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THE DETERMINATION OF POTASSIUM IN BLOOD SERUM.

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The present methods for the determination of potassium depend upon its precipitation as a sparingly soluble sodium-potassium cobalti-nitrite, and upon various procedures for estimating the latter. Kramer and Tisdall (1) precipitate the compound directly from serum and titrate it with permanganate. Doisy and Bell (2) and also Kerr (3) carry out the precipitation on a solution of the ashed residue from a trichloroacetic acid filtrate. The former introduced a sensitive colorimetric method of estimating the precipitate by diazotization after decomposing it to potassium nitrite, while the latter retained the permanganate titration. Fiske and Litarczek (4) ash the cobalti-nitrite precipitate and reprecipitate the potassium as the acid tartrate, which is titratable by alkali.

The authors encountered serious difficulties in applying the Kramer-Tisdall method to dog serum. The cobalti-nitrite precipitates when heated with 1 cc. of dilute (1:5) nitric acid left a yellow coagulum indicating protein. Frequent turbidity of dog sera from suspended fat introduced a further complication. Either ashing or precipitation of proteins would overcome both of these difficulties, but concentration of protein-free filtrates and ashing are time-consuming. Hence we were led to investigate the possibility of precipitating potassium as a potassium silver cobalti-nitrite. The latter was known to be far more insoluble than the corresponding sodium potassium salt (5, 6) and indeed proved satisfactory for direct precipitation of potassium from a slightly modified tungstic acid filtrate from which the chlorides have been removed.

For the quantitative estimation of the precipitate we employed

the reaction between salts of cobalt and ammonium thiocyanate in alcoholic solution. The color produced is blue, its intensity varying directly with the temperature and with the concentration of the alcohol. A quantitative study revealed that there is a sufficient deviation from direct proportionality to require construction of an empirical curve. Only a few minutes are required for the final estimation after precipitation and washing have been completed. As will be pointed out later, the procedure of Doisy and Bell can also be applied to estimate the precipitate obtained by our method.

Reagents.

1. Potassium Reagent.—To 20 cc. of sodium cobalti-nitrite reagent add 2 cc. of 40 per cent silver nitrate solution. Shake vigorously. Filter to remove the trace of precipitate which fails to redissolve. The sodium cobalti-nitrite reagent is prepared according to Kramer and Tisdall:

"*Solution A.*—25 gm. of cobalt nitrate crystals are dissolved in 50 cc. of water and to this solution are added 12.5 cc. of glacial acetic acid.

Solution B.—120 gm. of sodium nitrite (potassium-free) (Merck) are dissolved in 180 cc. of water. This gives a total volume of about 220 cc. To all of Solution A are added 210 cc. of Solution B. An evolution of nitric oxide gas occurs at once. Air is drawn through the solution until the gas has passed off. The reagent is placed in the ice chest and filtered each time before using. It will keep at least 1 month."

2. Dilute Nitric Acid.—A solution prepared by diluting 20 cc. of concentrated nitric acid (sp. gr. 1.42) to 100 cc. with water.

3. Ammonium Thiocyanate Solution.—A freshly prepared 2 per cent solution of the C.P. salt in 95 per cent alcohol.

4. Potassium Standard.—An aqueous solution of potassium sulfate containing 1.0 mg. of potassium per cc., prepared by dissolving 2.2276 gm. of the salt and diluting to 1 liter. This is a stock solution. For the standard dilute 5 cc. of this to 100 cc., making a solution containing 0.25 mg. of potassium in 5 cc.

5. Silver Nitrate Solution.—A 5 per cent aqueous solution.

6. Protein Precipitants.—A 10 per cent solution of sodium tungstate, and a $\frac{2}{3}$ N solution of sulfuric acid.

Procedure.

Place 2 cc. of serum in a 25 cc. test-tube. Add 5 cc. of water, and 1 cc. of sodium tungstate solution. Mix. Add 1 cc. of $\frac{2}{3}$ N sulfuric acid. Stopper and shake well. Add 1 cc. of the 5 per cent silver nitrate solution and shake again. After 15 minutes standing centrifuge vigorously. The precipitate should occupy considerably less volume than the supernatant liquid. The latter may be filtered through a very small ash-free paper or may be removed with a pipette. If one covers the top of the pipette with the finger any film on the surface of the liquid will adhere to the outside of the pipette. The tip of the pipette is brought to within about 4 mm. of the precipitate, and the pipette secured in position by being held diagonally in the tube by pressure with the thumb of the same hand which holds the tube.

In two 15 cc. graduated conical Pyrex centrifuge tubes, which have been cleaned previously with sulfuric acid-bichromate mixture,[1] place 5 cc. of the potassium standard (0.25 mg. of K) and 5 cc. of serum filtrate respectively. Pass the tube with the standard solution through the flame a few times so that it becomes barely warm to the hand.[2] Then add 2 cc. of the potassium reagent to each tube. After 2 hours standing centrifuge for 15 minutes at a speed of about 1200 revolutions per minute. With a capillary siphon, the tip of which bends upward, siphon off the supernatant liquid to the 0.3 cc. mark. Wash three or four times with 5 cc. portions of distilled water, as in the Kramer-Tisdall method. Centrifuge for 5 minutes each time, and siphon off the washing to the 0.3 cc. mark. The wash water should be mixed well with the residual solution above the precipitate, although the latter is stirred up as little as possible. The last washing should be free of yellow color. In the case of the last siphoning especial care should be taken to remove the supernatant liquid exactly to the 0.3 cc. mark. After removal of the wash water add 1 cc. of

[1] Tubes which have been cleaned and dried should of course be rinsed before being used to avoid any trace of ammonium salt, which also forms a highly insoluble cobalti-nitrite.

[2] The silver compound will otherwise precipitate very rapidly from standards containing over 0.15 mg. of potassium in 5 cc. and will form a fine yellow precipitate which adheres to the tube instead of a coarser one which is reddish after centrifuging.

the dilute nitric acid to each tube, and heat carefully to boiling over a micro burner. Just as boiling begins remove the tube from the flame and shake gently. In 10 to 20 seconds the yellow precipitate dissolves completely. Should any trace remain it can be dissolved by continuing the heating below the boiling point, and shaking several times. Cool the tubes under the tap, add alcoholic ammonium thiocyanate solution to the 8 cc. mark, mix, and compare in the colorimeter. All glass cups requiring only 3 cc. for a reading were used. It is well to rinse cups and plungers with alcohol acidified with nitric acid, before using them. As is the rule with alcoholic solutions, minute bubbles under the plunger must be watched for and removed.

Calculation.—Reference to the empirical curve gives the amount of potassium corresponding to the reading obtained. Since the equivalent of 1 cc. of serum was used, the same figure with the decimal point shifted two places to the right equals potassium in mg. per 100 cc. of serum.

Construction of the Empirical Curve.

From the stock solution of potassium sulfate containing 1 mg. of potassium per cc. prepare eight solutions containing 0.15 to 0.50 mg. of potassium per 5 cc., the solutions differing by 0.05 mg. per 5 cc. Carry out several series of determinations on these solutions, using 5 cc. of each, and taking the one containing 0.25 mg. of potassium as standard. The average readings obtained are plotted against the corresponding quantities of potassium. The curve obtained is shown in Fig. 1. The data for construction of the curve are given in Table I.

Recovery of Potassium Added to Serum.

In Table II are recorded the results of a number of experiments in which amounts of potassium ranging from 5 to 25 mg. per 100 cc. were added to 2 cc. portions of serum. The samples with and without addition were then carried through the procedure as outlined.

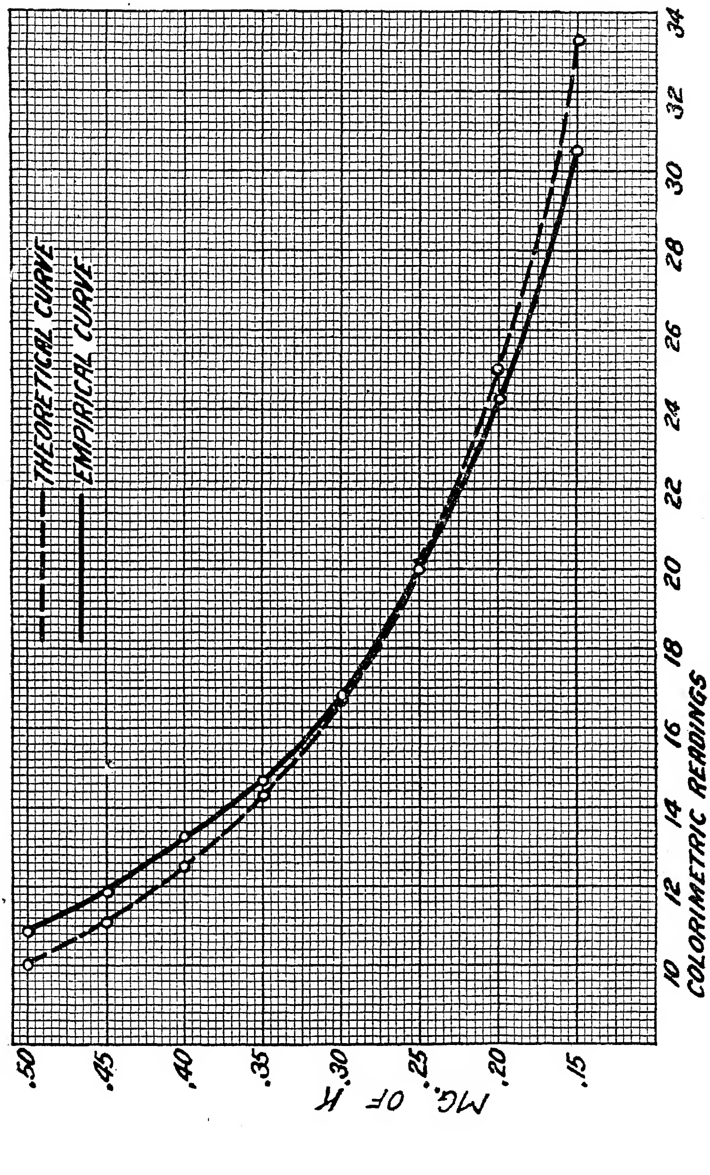

FIG. 1. Chart for calculation of results.

Comparison with Results Obtained by Ashing.

A series of samples of dog serum was ashed in silica beakers as follows: 5 cc. of serum, 3 cc. of reagent grade concentrated sulfuric acid, and 5 cc. of redistilled 60 per cent perchloric acid were

TABLE I.

Colorimetric Readings Corresponding to Given Amounts of Potassium.

Amount of K.	Colorimetric reading.
mg.	*mm.*
0.15	30.5
0.20	24.3
0.25	20.0
0.30	16.8
0.35	14.7
0.40	13.3
0.45	11.9
0.50	10.9

TABLE II.

Recovery of Potassium Added to Serum.

K added per 100 cc. serum.	K found per 100 cc. serum.	K recovered per 100 cc. serum.
mg.	*mg.*	*mg.*
0	15.1	
5	20.0	4.9
10	26.0	10.9
15	29.5	14.4
20	35.5	20.4
25	41.0	25.9
0	18.5	
5	23.5	5.0
10	28.5	10.0
15	33.5	15.0
20	40.0	21.5

placed in a 100 cc. tall form, 50 gm. silica beaker. This was heated on a sand bath at such a temperature that strong fuming but no splattering resulted. The residue was black, but after a second treatment with the same amounts of the acids became pure white. After visible acid had disappeared the tem-

perature of the sand bath was increased and heating continued for at least half an hour, to avoid crepitation during the subsequent heating. All parts of the beaker were then heated just to beginning redness over the free flame. After cooling, the ash was dissolved in 5 cc. of water and potassium determined in duplicate 2 cc. portions by the Kramer-Tisdall method. It was found that known amounts of potassium equivalent to the amount in the quantity of serum used could be carried through the procedure without loss, provided the amount of sodium chloride introduced with the serum was also added. The results presented in Table III take into account a very small blank for potassium in the sulfuric acid used in ashing, determined in connection with the recovery of potassium from pure solutions.

TABLE III.

Comparison of Results Obtained with and without Ashing.

Sample No.	K in 100 cc. serum.	
	Authors' method, without ashing.	Kramer-Tisdall method, after ashing.
	mg.	*mg.*
1	17.0	17.1
2	17.8	17.0
3	19.0	18.2
4	18.5	17.1
5	18.5	18.4
6	17.8	16.3
7	16.5	16.4
8	19.5	18.5

In Table III are given the results obtained by this ashing procedure, and parallel with them the results obtained on 2 cc. portions of the same sera by our method. The results tabulated are consecutive, without exclusions. In a few cases the discrepancy between the direct result and that after ashing is more than 1 mg. of potassium per 100 cc. of blood, but the average value is 0.7 mg. lower by the ashing process. On consideration of the fact that the amounts actually determined are multiplied by 50 in one case and 100 in the other to obtain the values per 100 cc. of blood, this difference is not large.

Use of Other Methods of Estimating the Potassium Silver Cobalti-Nitrite Precipitate.

Due to the presence of silver the precipitate obtained in our method cannot be titrated with permanganate. It can be estimated gasometrically in a manner similar to that of Kramer and Gittleman (7). The method of Doisy and Bell is also applicable, and its use in this connection combines the most complete precipitation of potassium with the most sensitive color reaction available for the final measurement. This enables one to carry out the estimation with filtrate equivalent to 0.5 cc. of serum, as follows: The filtrate is prepared from 1 cc. of serum, 7.5 cc. of water, 0.5 cc. of 10 per cent sodium tungstate, 0.5 cc. of $\frac{2}{3}$ N sulfuric acid, and 0.5 cc. of 5 per cent silver nitrate solution. Precipitation and washing are carried out in the manner described above, with 5 cc. of filtrate, and, as a standard, 5 cc. of potassium sulfate solution containing 0.08 mg. of potassium in this volume. After the washing has been completed 5 cc. of 0.2 N sodium hydroxide solution are added to each tube. The contents are heated to boiling, filtered into 50 cc. volumetric flasks, and made up to volume by washing. 8 cc. portions of standard and unknown are transferred to 100 cc. volumetric flasks, and diluted to about 70 cc. 2 cc. of sulfanilic acid solution (0.5 per cent in 30 per cent acetic acid) and 1 cc. of naphthylamine solution (0.5 per cent in 30 per cent acetic acid) are added. After dilution to volume and 10 minutes standing comparison is made in the colorimeter. The formula $\frac{\text{reading of standard}}{\text{reading of unknown}} \times 16$ gives the value in mg. of potassium per 100 cc. If the reading is too far from the standard there is ample solution left to repeat the final determination with a different aliquot of the contents of the 50 cc. volumetric flask.

SUMMARY.

1. A method is described for direct precipitation of potassium from serum filtrate. This avoids errors due to proteins and fat without requiring concentration of filtrates or ashing.

2. One of the color reactions employed for estimating the precipitate is also of interest as an additional one for the determination of small amounts of cobalt.

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STUDIES OF THE METABOLISM OF WOMEN.

IV. THE CALCIUM AND INORGANIC PHOSPHORUS IN THE BLOOD OF NORMAL WOMEN AT THE VARIOUS STAGES OF THE MONTHLY CYCLE.

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That the activities of the female reproductive tract may exert some influence on calcium metabolism has been indicated in various ways.

Mirvish and Bosman (1) have reported that they can produce by injection of ovarian follicular and corpus luteum extracts in rabbits and in human beings, a 2 to 3 mg. lowering of plasma calcium. Luckhardt and Goldberg (2) have demonstrated that estrus will cause a return of tetany in parathyroidectomized dogs in which the tetany has previously been controlled by administration of calcium lactate.

Because menstrual cramps have been supposed to be associated with variations in calcium in the uterine blood supply and musculature, considerable attention has been paid to investigations of the calcium level in the blood at various phases of the menstrual cycle. Reference is made to the more important papers in the bibliography (3–13). With the exceptions noted below, these papers are based upon tabulations of one or two menstrual and one or two intermenstrual figures for any one person. The subjects used have been, to a large extent, pathological cases, and the number of observations has been too small to justify the variable conclusions drawn.

The three papers of Sharlet, Corscaden, and Lyle (11), Bock (12), and Matters (13) all of which have appeared since the present investigation was under way, have presented series of observations taken at more frequent intervals, but on very small numbers of

subjects. The first two of these papers conclude that there is a tendency for slightly higher levels of circulating calcium to occur just preceding menstruation, while Matters considers that the onset of menstruation is accompanied by a lowering of blood calcium.

While there is abundant evidence in the literature that the concentration of calcium and phosphorus in the blood stream are in some way related we have been able to find only the very limited observations of Close and Osman (14) on menstrual variations in blood phosphates. They conclude that menstruation produces no change in phosphorus level.

EXPERIMENTAL.

Our study of blood calcium and phosphorus in relation to the menstrual cycle in women was undertaken as a part of a general plan of study of the conditions associated with menstrual discomfort and loss of efficiency in otherwise normal young women. The subject appealed to us as especially interesting because of the association of disturbances in calcium and phosphorus metabolism with other conditions of increased irritability of muscle and nerve and with disturbances in coagulability of blood. We were also interested in the possible basis for the somewhat indiscriminate clinical use of calcium lactate as a therapeutic in menstrual disorders.

Methods and Procedure.

The figures for *serum* calcium included in Table I represent determinations by a Kramer-Tisdall procedure (15) modified to the extent of allowing 3 hours at ice box temperature for the original precipitation of the Ca oxalate, and washing with water which had previously been allowed to stand in contact with solid Ca oxalate and then filtered through a hard filter (16).

A series of 240 determinations of plasma calcium by the Clark (17) procedure which we have made on blood from fourteen women taken three times a week throughout a total of eighteen monthly cycles is considered in the discussion but not reported in detail because of lack of space.

Inorganic phosphorus was determined by the method of Fiske and Subbarow (18). Total acid-soluble phosphorus was deter-

TABLE I.

Before Breakfast Serum Calcium and Inorganic Phosphorus in Relation to the Menstrual Cycle.

Date.*	Day in relation to menses.	Ca	P
NT. Age, 30 yrs.; height, 62.5 in.; weight, 138 lbs.; 25 day cycle.			
1928		*mg. per 100 cc.*	*mg. per 100 cc.*
Sept. 3	−16	10.5	4.1
" 5	−14	10.6	4.7
" 7	−12	10.1	4.1
" 10	−9	10.5	3.7
" 12	−7	10.2	3.7
" 14	−5		3.7
" 17	−2	10.8	3.9
" 19	0	10.2	3.7
" 20	+1	10.2	3.5
" 21	+2	10.0	3.6
" 24	+5	10.3	
" 26	+7	10.1	4.0
" 28	+9	10.1	
Average..........		10.3	3.9
EX. Age, 22 yrs.; height, 61 in.; weight, 137 lbs.; 25 day cycle.			
Nov. 16	−3	10.1	3.5
" 19	0	10.3	4.0
" 21	+2	9.8	
" 23	+4	10.6	3.8
" 28	+9	10.5	3.8
Dec. 3	+14	10.5	4.0
" 5	−10	10.4	
" 7	−8	10.1	3.5
" 15	0	10.3	3.1
" 17	+2	10.5	3.4
" 19	+4	10.6	
Average..........		10.3	3.6
NN. Age, 23 yrs.; height, 62.5 in.; weight, 138 lbs.; 28–33 day cycle.			
1928		*mg. per 100 cc.*	*mg. per 100 cc.*
Sept. 19	−4	10.1	3.6
" 21	−2	9.7	3.4
" 24	+1	9.6	4.0
" 26	+3	10.1	4.2
" 28	+5	9.5	3.8
Oct. 1	+8	10.1	3.7
" 3	+10	9.7	3.8
" 5	+12	9.9	4.0
" 8	+15	9.7	3.3
" 10	+17	10.0	3.8
" 12	+19	9.6	3.6
Average..........		9.8	3.7
EE. Age, 21 yrs.; height, 66.5 in.; weight, 140 lbs.; 25–28 day cycle.			
Apr. 26	−1	10.1	3.1
" 28	+1	10.4	3.1
May 1	+4	10.1	2.8
" 2	+5	10.0	2.6
" 7	−15	10.2	3.2
" 9	−13	10.8	3.5
" 12	−10	10.6	3.0
" 15	−7	10.8	2.9
" 17	−5	10.3	3.0
" 21	−1	10.2	3.1
" 22	0	9.6	3.8
" 23	+1	8.7	3.4
" 25	+3	9.8	3.0
" 28	+6	9.7	3.0
Average..........		10.1	3.1

* Days before the onset of menstruation are listed as (−), days after as (+), the day of onset as 0.

TABLE I—*Continued.*

Date.*	Day in relation to menses.	Ca	P
BS. Age, 23 yrs.; height, 64 in.; weight, 120 lbs.; 28 day cycle.			
1928		*mg. per 100 cc.*	*mg. per 100 cc.*
Oct. 5	−20	10.5	3.9
" 8	−17	10.2	4.9
" 10	−15	10.0	3.4
" 12	−13	10.6	4.0
" 15	−10	10.8	4.0
" 17	−8	10.5	3.8
" 19	−6	10.9	3.8
" 22	−3	9.7	3.8
" 24	−1	10.1	4.0
" 25	0	**10.6**	**4.1**
" 26	+1	**10.7**	**4.5**
" 29	+4	**10.5**	**4.2**
" 31	+6	10.4	4.3
Nov. 2	+8	10.4	4.6
Average.........		10.4	4.1
FD. Age 27 yrs.; height, 63 in.; weight, 115 lbs.; 26 day cycle.			
Oct. 22	0	10.6	3.5
" 23	+1	**10.0**	**3.8**
" 24	+2	**10.2**	**4.1**
" 26	+4	9.6	3.9
" 29	+7	10.1	3.7
" 31	+9	10.0	3.8
Nov. 2	+11	10.1	3.9
" 5	−12	9.9	3.2
" 7	−10	9.9	3.6
" 9	−8	10.2	
" 14	−3	9.9	3.3
" 16	−1	10.1	3.8
" 18	+1	**10.1**	**4.0**
" 19	+2	10.1	3.9
" 21	+4	9.9	
" 23	+6	10.3	3.4
Average..........		10.07	3.7
FD.—continued.			
1929		*mg. per 100 cc.*	*mg. per 100 cc.*
May 7	+8	9.5	3.4
" 9	−15	10.0	3.0
" 12	−12	10.7	3.0
" 15	−9		3.1
" 17	−7	10.6	3.3
" 21	−3	10.3	2.9
" 23	−1	9.7	2.9
" 24	0	**9.9**	**3.0**
" 25	+1	**10.3**	**3.5**
" 29	+5	**10.0**	**3.2**
Average.........		10.10	3.14
BG. Age, 26 yrs.; height, 60 in.; weight, 106 lbs.; 28 day cycle.			
1928			
Nov. 19	0	**10.0**	**3.9**
" 20	+1	**10.2**	**3.8**
" 21	+2	**10.0**	**4.3**
" 23	+4	**10.1**	
" 26	+7	9.9	3.6
" 28	+9	10.4	4.1
Dec. 1	+12	10.2	
" 3	+14	10.0	3.8
" 5	−12	10.2	4.7
" 7	−10	10.5	4.4
" 10	−7	10.2	3.5
" 12	−5	10.0	4.3
" 14	−3	10.2	4.5
" 17	0	**10.2**	**4.4**
" 18	+1	**10.4**	
" 21	+4	**10.3**	**3.8**
Average.........		10.2	4.1

TABLE I—*Continued.*

Date.*	Day in relation to menses.	Ca	P
IG. Age, 24 yrs.; height, 67 in.; weight, 119 lbs.; 28 day cycle.			
1928		*mg. per 100 cc.*	*mg. per 100 cc.*
Mar. 27	−6	9.6	3.2
" 29	−4	9.7	2.9
" 30	−3	9.8	3.2
Apr. 3	+1	9.6	2.8
" 5	+3	9.0	2.8
" 6	+4	8.6	3.0
" 10	+8	8.9	3.0
" 12	+10	9.3	3.3
" 14	+12		3.0
" 17	+15	9.0	2.9
" 19	−13	9.3	3.2
" 24	−8	9.7	3.1
" 26	−6	9.2	3.0
" 28	−4	9.2	2.9
May 1	−1		2.8
" 2	0	9.9	2.9
" 4	+2	10.0	2.9
Average.........		9.6	3.0
FM. Age, 30 yrs.; height, 60 in.; weight, 117 lbs.; 28 day cycle.			
Oct. 13	0	9.9	3.3
" 14	+1	9.9	2.9
" 15	+2	10.1	3.6
" 17	+4	9.9	3.6
" 19	+6	10.3	3.3
" 22	+9	9.8	4.0
" 24	+11	10.0	3.2
" 26	+13	9.9	2.8
" 29	−11	9.9	4.0
" 31	−9	9.9	3.3
Nov. 2	−7	9.6	3.3
" 5	−4	9.8	3.6
" 7	−2	9.9	2.9
" 9	0	9.5	3.5
" 10	+1	9.5	3.4
" 11	+2	9.5	3.1
" 14	+5	9.8	3.5
Average.........		9.8	3.35

Date.*	Day in relation to menses.	Ca	P
MK. Age, 21 yrs.; height, 70 in.; weight, 165 lbs.; 24–28 day cycle.			
1928		*mg. per 100 cc.*	*mg. per 100 cc.*
Mar. 27	−20	9.5	3.3
" 29	−18	9.6	3.2
" 31	−16	9.5	3.3
Apr. 3	−13	8.9	3.0
" 5	−11		2.8
" 6	−10	9.6	3.4
" 10	−6	9.4	3.4
" 12	−4	8.6	3.2
" 14	−2	9.0	3.0
" 17	+1		3.2
" 19	+3	9.5	
" 20	+4	9.8	3.1
Average.........		9.3	3.2
SC. Age, 29 yrs.; height, 63 in.; weight, 138 lbs.; 28 day cycle.			
Sept. 3	−11	10.0	3.9
" 5	−9	10.5	3.8
" 7	−7	10.4	3.6
" 10	−4	10.2	3.3
" 12	−2	9.9	3.6
" 14	0		3.5
" 15	+1	10.2	4.0
" 17	+3	9.9	3.0
" 19	+5	10.3	3.3
" 21	+7	10.3	3.3
" 24	−16	9.7	3.1
" 26	−14	10.4	3.2
" 28	−12	10.4	4.7
Oct. 1	−9	10.0	3.4
" 3	−7	10.5	2.8
" 5	−5	10.1	3.0
" 8	−2	9.9	3.1
" 10	0	10.3	3.4
" 11	+1	10.6	3.0
" 12	+2	10.0	3.5
" 15	+5	9.9	3.3
" 17	+7	10.3	3.4
Average.........		10.14	3.4

TABLE I—*Concluded.*

Date.*	Day in relation to menses.	Ca	P
MO. Age, 20 yrs.; height, 64 in.; weight, 130 lbs.; 28 day cycle.			
1928		*mg. per 100 cc.*	*mg. per 100 cc.*
Sept. 24	−11	10.4	4.4
" 26	−9	10.3	4.0
" 28	−7	10.0	3.7
Oct. 1	−4	10.4	
" 3	−2	10.3	4.1
" 5	0	10.2	4.0
" 8	+3	10.3	3.8
" 10	+5	10.7	3.6
" 12	+7	10.7	3.8
" 15	+10	10.0	4.0
" 19	+14	10.5	3.7
" 22	+17	10.6	4.2
" 24	+19	10.0	4.2
Average.........		10.3	4.0

mined by combustion of the trichloroacetic acid filtrate followed by colorimetric estimation of the phosphate by the above procedure.

The subjects used were students, normal in so far as could be determined from their records at the University Infirmary. The blood samples were taken before breakfast according to the general procedure described in the first paper of this series (19).

Diets were not restricted but the subjects were instructed to limit themselves to as nearly a constant food intake as their boarding house regimens would permit, during the period of observation.

DISCUSSION.

It is apparent from the data in Table I, that there is no very marked or consistent variation in serum calcium or phosphorus at any phase of the monthly cycle. There seems to be some tendency to frequency of higher values for calcium between 10 and 15 days following the onset of menstruation, possibly at the time of rupture of the Graafian follicles. There is also some tendency to

frequency of lower values during the days immediately preceding onset of menstruation presumably at the time of discharge of the maximal quantities of the hormone of the corpus luteum. These tendencies are rather more evident in the figures for plasma than in those for serum (Table I).

Statistical treatment of the data is made difficult by the inequality of intervals between menstrual periods in different individuals. Differences in individual time relationships between ovulation and menstruation still further complicate the situation, because we have no outward manifestation of ovulation and it would even seem to be fairly generally conceded that ovulation does not necessarily have to precede menstruation. Moreover, we have taken our samples three times a week only, and while we believe this is as often as may very well be done without introducing additional sources of error due to the effects of repeated bleeding, nevertheless, the lack of daily observations still further complicates the problem of statistical evaluation of data.

We have divided our figures for each menstrual interval into 3 day periods and computed, for each period, the standard deviation and probable error of all our data on serum calcium and phosphorus. These indicate that, considered from a purely mathematical point of view, the variations in serum calcium at the periods probably corresponding to the times of ovulation and to the maximal production of the hormone of corpus luteum are just smaller than those necessary to be considered absolutely significant. It has seemed impossible to determine whether this may be due to individual differences in the intervals between menstruation and ovulation, to the inclusion of cases in which menstruation has occurred at an irregular interval after ovulation, or to variability in serum calcium and phosphorus produced by entirely extraneous causes. An attempt has also been made to evaluate the data on serum calcium and phosphorus and on plasma calcium by making composite curves, the figures being spaced in terms of days from the onset of menstruation and a 10 point moving average used. These are given in Chart I.

Mathematically considered, while the variations in calcium of citrated plasma are greater from day to day, nevertheless they seem more significant than those in serum calcium. This suggests that the hormone of corpus luteum may exert a greater

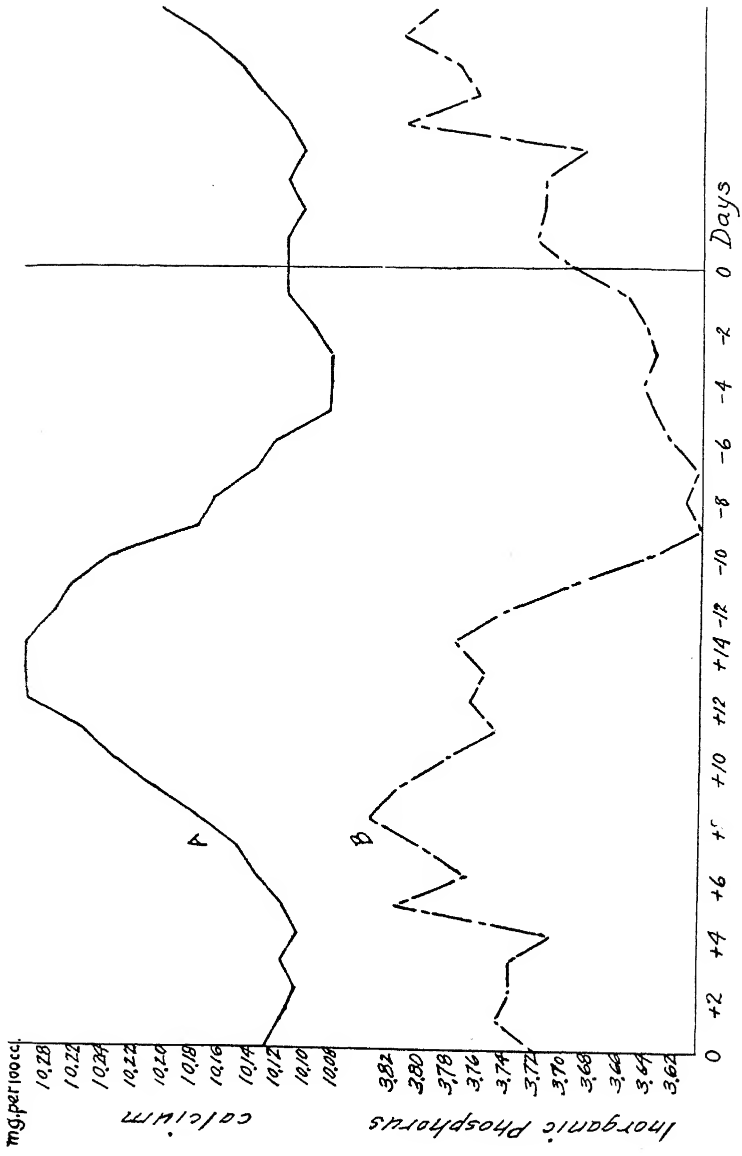

CHART I. Composite curves of serum calcium and inorganic phosphorus.

TABLE II.

Data for Men.

Plasma Values.

Subject Qm. Age, 25 yrs.; height, 69 in.; weight, 163 lbs.		Subject Cf. Age, 22 yrs.; height, 70 in.; weight, 135 lbs.		Subject Mf. Age, 38 yrs.; height, 68½ in.; weight, 160 lbs.		Subject Sb. Age, 39 yrs.; height, 68 in.; weight, 140 lbs.	
Date.	Ca	Date.	Ca	Date.	Ca	Date.	Ca
1926	*mg. per 100 cc.*	*1926*	*mg. per 100 cc.*	*1926*	*mg. per 100 cc.*	*1926*	*mg. per 100 cc.*
Apr. 5	9.0	Apr. 9	8.6	Jan. 20	9.3	Apr. 7	9.2
" 7	9.0	" 12	8.8	" 22	9.5	" 9	9.0
" 9	9.0	" 14	8.6	" 25	9.6	" 12	8.9
" 12*	8.2*	" 16	8.9	" 27	9.6	" 14	8.9
" 14*	8.6*	" 19	8.7	" 29	9.2	" 16	8.9
" 16*	8.7*	" 21	9.0	Feb. 1	9.2	" 19	8.8
" 19	8.7	" 23	9.0	" 2	9.2	" 21	8.8
" 21	8.5	" 26	8.7	" 5	9.3	" 23*	8.7*
" 26	8.7	" 28	8.6	" 8	9.3	" 26*	8.4*
" 28	8.7			" 10	9.3	" 28	9.1
				" 12	9.4		

Serum Values.

Subject Xd. Age 28 yrs.; height, 65 in.; weight, 121 lbs.			Subject DI. Age 25 yrs.; height, 68 in.; weight, 165 lbs.		
Date.	Ca	P	Date.	Ca	P
1928	*mg. per 100 cc.*	*mg. per 100 cc.*	*1928*	*mg. per 100 cc.*	*mg. per 100 cc.*
Oct. 22	10.5	3.8	Nov. 26		3.6
" 24	10.2	3.8	" 28	10.4	3.5
" 26	10.1	4.2	Dec. 3	10.2	3.8
" 29*	9.7*	4.3	" 5	10.3	3.5
" 31*	9.9*	3.8	" 7	10.7	3.5
Nov. 2*	9.6*	4.3	" 10	10.7	3.6
" 5	9.9	3.6	" 12		3.3
" 7	10.1	4.0	" 14	10.3	3.8
" 9	10.1	3.6	" 17	10.4	3.7
" 14	10.1	3.6			
" 16	9.9	3.8			
" 19	10.2	3.7			
" 21	10.1				

* Subjects suffering from respiratory infection.

influence on the state of calcium than on actual concentration of calcium in the blood. It is interesting to note that the figures by Sharlet, Corscaden, and Lyle (11), who used plasma for their study, show about the magnitude of variation characteristic of our figures for citrated plasma—while those of Bock (12) correspond to our figures for serum; also that Mirvish and Bosman were estimating plasma calcium where they reported that it was lowered by the administration of ovarian extracts.

We have attempted to measure the variations in blood calcium in men for control data. The figures shown in Table II indicate a rather smaller day to day deviation from the mean values for the individuals in men than in women. Indeed, if we rule out calcium determinations made when the subjects were known to be suffering from colds, sore throat, etc., the greater constancy of the values for men would seem to be significant. Unfortunately, the number of men subjects available for this study was too small to justify mathematical comparison of data. We greatly regret that we were unable to secure women past the age of menopause as control subjects.

One would probably expect the variations in calcium of circulating blood at the time of menstruation to be much smaller than those in the menstrual discharge or in the calcium of the menstruating endometrium. The difficulties involved in collecting in normal women menstrual blood uncontaminated by vaginal secretion, or in securing other than grossly pathological tissue specimens from the operating room, stand in the way of direct investigation of the calcium content of these tissues. It is obvious that such data are needed.

The data on inorganic phosphorus show a by no means consistent relationship between the concentration of this substance and that of the serum calcium. There is some tendency to frequency of low values for serum phosphorus at about the 8th to 10th days preceding the onset of menstruation and to an increase in concentration of phosphorus at about the 4th to 6th days of the menstrual period. Considered from a purely mathematical point of view, these variations in concentration of inorganic phosphorus are probably too irregular and of too small magnitude to be significant. But the composite curve (Chart I) at least suggests that inorganic phosphate may be used up in the synthesis of

lecithin in the uterine mucosa during the stage of pregestational or premenstrual proliferation and that the menstrual breakdown of the endometrium may be accompanied by decomposition of lecithin and some resorption of phosphate. It will obviously be necessary to do more work on tissue composition before this point can be established.

Total acid-soluble phosphorus was determined throughout three menstrual periods in three different individuals. Values ran very nearly parallel to those for inorganic phosphorus, and about 1 mg. per cent higher. These figures are not given in detail because of the space required for the tables.

This work has been made possible by several grants from the University Board of Research and by a grant from the Committee for Research in Problems of Sex of the National Research Council.

We wish to acknowledge also our indebtedness to Dr. Lillian Moore (deceased August, 1929) for her kindly assistance in evaluation of the data, and to Dr. Sylvia Parker for criticism of the statistical work.

SUMMARY AND CONCLUSIONS.

Figures for before breakfast values for serum calcium and phosphorus determined three times weekly in twelve women for periods averaging 5 weeks each are reported, together with similar figures for six men. A similar series of observations on plasma calcium in fourteen women is also discussed.

While the changes in concentration of serum calcium at any phase of the monthly cycle are not outstanding, there is some tendency to frequency of low values for calcium a few days previous to the onset of menstruation and to frequency of higher values from the 8th to the 15th days following the onset of menstrual bleeding.

Day to day variations of serum calcium are smaller for men than for women.

Variations in serum phosphorus are less consistent but our data at least suggest that the period of greatest proliferation of uterine mucosa preceding menstruation may be associated with a using up of inorganic phosphous from the blood and that some extra inorganic phosphorus may be thrown into the blood stream following menstrual destruction of endometrial tissue.

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FAT-SOLUBLE VITAMINS.

XXVIII. THE ANTIRACHITIC VALUE OF COW'S MILK AS MODIFIED BY EXPOSURE OF THE COW TO SUNLIGHT AND TO RADIATIONS FROM A QUARTZ MERCURY VAPOR LAMP.*

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While the seemingly intricate relations of the growth-promoting and antirachitic properties of light and cod liver oil were being unravelled, Luce (1) studied the influence of insolation and feed of the cow upon the antirachitic properties of milk. She concluded that the effect depended upon the diet of the cow, and possibly on the degree of insolation. In a second paper she (2) stated that the diet of the cow appeared to be the main factor in determining the antirachitic value of the milk, because, with a diet deficient in the fat-soluble vitamins, action of sunlight alone failed to raise the antirachitic potency materially.

The work of Luce was elaborated upon by Boas and Chick (3). Milk from a cow kept in a dark stall on dry or fresh green fodder was deficient in calcium-depositing properties, but milk produced on pasture induced normal bone formation. They were inclined to attribute this difference in quality to the effect of sunlight upon the cow.

Later, Chick and Roscoe (4) availed themselves of refined methods of analysis based on the different effects produced by vitamins A and D respectively, and concluded that the antirachitic value of milk depended principally upon the degree of insolation of the cow. At the same time, they felt that there was some indication that the diet of the cow was a contributing factor.

Besides the work of the Lister experimenters, similar studies were carried out in other laboratories. Falkenheim, Völtz, and coworkers (5–7) found

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that the milk of a cow irradiated with ultra-violet light, in contrast with milk from a non-irradiated cow, showed itself to be antirachitically active, prophylactically and curatively. Sunlight did not produce definite effects.

Gowan and coworkers (8, 9) found 15 minutes of irradiation daily with a Cooper Hewitt lamp at 3 feet from the cow sufficient to increase the antirachitic potency of milk, as determined by feeding tests on rachitic chickens.

Dutcher and Honeywell (10) reported that butter from cows subjected to sunlight possessed superior calcifying properties over butter produced in the dark when fed to rats.

Hess, Weinstock, and Sherman (11) demonstrated the appearance of vitamin D in milk by irradiation of the mother.

Hess and Unger (12) reported that summer-produced milk powder failed to prevent rickets when fed during the winter. However, Supplee and Dow (13) found that summer-produced milk powder possessed greater antirachitic properties than winter-produced milk powder.

Bruck-Biesok, Pirquet, and Wagner (14) irradiated cows with the Hanauer quartz mercury vapor lamp and found that the milk was sufficiently potent antirachitically to prevent rickets in children normal at birth. Florid cases of rickets could not be cured. Commenting on the pronounced results of Völtz and coworkers, they state that their less effective results were probably caused by less intensive irradiation.

Gerstenberger, Hartman, and Smith (15) reported that human milk from mothers irradiated with artificially produced ultra-violet light gave evidence of possessing definite antirachitic properties (16).

Our interest in this problem dates from 1923, when we made our first observations on the antirachitic activation of organic substances; but it was not until 1925 that we carried out experiments on the indirect activation of milk by irradiation of the cow. At about this time Professor Dougherty also showed at this station that the antirachitic value of egg yolk could be increased by irradiation of the laying hen (17). This suggested to us that other secretions of the animal body possibly could be changed in character as well.

For our lactation experiments, we used a goat as the experimental animal and controlled our results by feeding irradiated cow's milk which we had previously shown could be directly activated (18), at the same time feeding goat's milk directly activated, untreated, and as obtained from the irradiated animal.

We pointed out that Luce (1, 2) might have been unwarranted in her conclusion that the feeding of an animal was a more important determinant of the antirachitic properties of the milk than the extent of exposure, to ultra-violet light, of the animal itself. Our

data on the effect of hays cured in the sun (19) had emphasized the variability of the calcifying action of feeds. This apparently depended upon their exposure to ultra-violet radiations. The outcome of our goat experiments was a demonstration of the fact that excluding the feed as a possible factor in determining the antirachitic potency of milk, which had been done in the experiments of Luce (1, 2), irradiation, at least with the goat, produced marked increase in the antirachitic properties of milk. However, our experiments differed further from those of Luce (1, 2) in that we used the intense ultra-violet radiations of a quartz mercury vapor lamp instead of sunlight. As a result of our experiments we were very optimistic over the possibilities of improving cow's milk in a similar manner, and called attention to the possible economic and hygienic relations of the results which we obtained to the proper maintenance of dairy herds and the production of a better food for man.

EXPERIMENTAL.

Series 1. Insolation Experiments of 1925–26.

In our work of 1925–26 with cows of which the data on calcium balances have already been published (20) we accordingly made simultaneous observations on the change in antirachitic value of the milk produced. These observations, bringing out a comparison between milk produced by cows confined in the barn, with milk produced during brief insolation, and again with exposure during the entire summer, gave results differing much less in magnitude than previous work with goats had led us to expect. They have been commented on briefly (21).

Three pure bred Holsteins, Cows 1, 2, and 3 (the same animals as were used in our previous publication (20)), were used for the trials. They were kept confined in the barn without exposure to sunlight or sky light from January 1 to May 26, 1925. Then they were exposed to sunlight for 3 weeks for a total of $109\frac{1}{2}$ hours of actual sunshine from May 26 to June 16, and finally they were turned into the general herd which was out-of-doors continuously except from about the middle of July to the latter part of August, when they were kept in the barn owing to excessive heat.

The intensity of the radiation received by the cows cannot be expressed in a satisfactory manner because we have no data on the per cent of the total solar radiations which consist of ultra-violet nor specifically of the quantitative relations of those ultra-violet rays which are capable of effecting antirachitic activation; but, in lieu of that, we obtained from Dr. Eric Miller of the local United States Meteorological Station figures on the solar radiation excepting those rays absorbed by glass as obtained with a Callendar volumetric recorder. These data, presented in Table I, we hope may help in estimating approximate ultra-violet values if our results should later merit further analysis.

TABLE I.

Meteorological Records. May 26–June 17, 1925, Madison.

Date.	Hrs. of sunshine.	Percentage of total.	Calories per day per sq. cm.	Appearance of sky.
May 26	12.1	81	677	Partly cloudy.
" 27	4.1	27	352	Cloudy.
" 28	11.5	77	572	Partly cloudy.
" 29	15.1	100	613	Clear.
" 30	13.5	89	645	"
" 31	13.5	89	582	"
June 1	10.8	71	592	Partly cloudy.
" 2	9.8	64	469	Cloudy.
" 3	6.5	43	332	"
" 4	12.0	79	604	"
" 5	15.2	100	673	Partly cloudy.
" 6	15.2	100	654	Clear.
" 7	10.6	70	514	Partly cloudy.
" 8	14.0	92	626	" "
" 9	12.7	83	736	Clear.
" 10	11.6	76	631	Partly cloudy.
" 11	4.4	29	343	Cloudy.
" 12	7.4	48	454	"
" 13	13.6	89	671	Partly cloudy.
" 14	6.8	44	352	Cloudy.
" 15	10.0	65	439	"
" 16	14.8	96	636	"

During the sunlight period, the cows were taken out of the barn and exposed to the out-of-doors for a period of 6 hours beginning at 9 a.m. In the records of the local Station no account was taken

of the diurnal distribution of solar radiation, but it was calculated by Dr. Miller that approximately 64 per cent of the total daily radiation occurred between the hours of 9 a.m. and 3 p.m., when the cows were exposed.

Hess has shown that the pigmentation of the animal is a factor in the efficiency with which ultra-violet radiations effect antirachitic action (16). Anticipating a pronounced effect with our cows, we noted the extent to which their skin was marked with black pigment. It was observed that about one-fourth of the back of Cow 1 was pigmented, about five-sixths of that of Cow 2, and none of the back of Cow 3. Viewed from the rear with the udder visible, Cow 1 had only a few black spots, Cow 2 was mostly black, and Cow 3 again had only a few small black spots. The rest of the visible areas were white.

For the determination of the comparative antirachitic value of the milk, rats were used as the experimental animals. They were raised under our standard laboratory conditions and put on our rickets-producing Ration 2965, consisting of yellow corn 76, wheat gluten 20, calcium carbonate 3, sodium chloride 1 (22), at an approximate weight of 60 gm. After 3 to 4 weeks, when rickets had developed to a uniformly severe degree, they were transferred to individual cages and given daily doses of milk ranging from 1 to 12 cc. which was fed separately in small glass dishes as a supplement to their basal diet. Daily records of consumption of their basal rations were kept, and, if for any reason either Ration 2965 or milk was poorly consumed, observations on such animals were discarded. This happened but rarely, although frequently little of the basal ration was consumed the 1st day after the animals had been put into their individual cages, and quite frequently difficulty was experienced in getting the rats to consume such large supplements of milk as 12 cc. daily.

Usually the milk was fed fresh, although in some cases it was fed after it had been sterilized for ½ hour at 15 pounds pressure and then kept in the refrigerator. We questioned the justifiability of this sterilization, but we were led to believe from some tests carried out by the Johns Hopkins technique (23) that it would not affect the antirachitic properties of the milk.

After 10 days of milk feeding the rats were killed with ether and

the distal ends of the radii and ulnæ examined for recent mineral deposition by staining with silver nitrate. The results of these various trials are presented in Table II. Each lot in Table II gives the results obtained with an individual litter.

It will be noted in Table II that some data are also presented on Cows 4, 5, and 6. These were cows taken from the general herd which had been out on pasture from 6.30 a.m. to 2 p.m. from the middle of May to the middle of July. From then on until the latter part of August, they were kept inside during the day.

TABLE II.

Solar Irradiation and Antirachitic Properties of Cow's Milk.

Milk.	Pre-sunlight (May).			Sunlight (June).			End of summer (September).					
	Cow 1. Lot 3287.	Cow 2. Lot 3289.	Cow 3. Lot 3299.	Cow 1. Lot 3333.	Cow 2. Lot 3335.	Cow 3. Lot 3338.	Cow 1. Lot 3459.	Cow 2. Lot 3460.	Cow 3. Lot 3461.	Cow 4. Lot 3468.	Cow 5. Lot 3475.	Cow 6. Lot 3476.
cc.												
1	–	–	–	–		–	–	–	–	–	–	–
2	–	–	–	–	–	–	–	–	–	–	–	–
4	–	–	–	–	–	–	–	–	–	–	–	–
6	–	–	–	–	–	–	+	–	+	–	+	+
8	–	+	?	–	?	?	+	+	++	++	+	+
12	–	++	?	+	+	+	++		+++		++	+++

? = specks of calcium deposited in metaphyses.
– = negative test.
\+ = narrow line of calcium.
++ = medium "
+++ = wide "

Later they were turned out as before. Cow 4 was a pure bred Guernsey of a solid red-fawn color; Cow 5 was a dark Jersey; Cow 6 was a pure bred Holstein with a back practically all white. Their daily milk yields were respectively, 28, 26, and 18 pounds. The tests were carried out in the latter part of September and the beginning of October, 1925. Cow 4 had freshened September 20, Cow 5 February 11, and Cow 6 July 4, 1925. Cow 4 weighed 1140 pounds, Cow 5 1060 pounds, and Cow 6 1346 pounds.

Our data suggest that sunlight acting upon the cow directly has no effect in increasing the antirachitic value of the milk. With

milk taken from the cows during the sunlight period, at times with a sunlight exposure ranging from 55 hours, when samples were first taken, to 109½ hours later at the conclusion of the experiment, no increase was demonstrable. However, by the end of the summer an increase in antirachitic potency is definitely indicated because 6 cc. were apparently as efficient as 12 cc. taken in the preliminary pre-sunshine period. The weakness in our experiment lies in the fact that when the cows were continued on the same ration, no effect of insolation could be demonstrated; but later on in the summer, during which time it was necessary to change the ration of the animals, it was impossible to say whether the increase observed was due to the direct effect of light upon the animal or the indirect effect produced through the ration. Outside of this the difficulties of quantitative testing for antirachitic values with this method are also probably more involved than those usually encountered.

When it comes to determine the antirachitic value of such a material as milk from time to time, it is necessary to have the test animals in a comparable receptive state. This is by no means a simple matter; but, in general, we have found that the gross behavior of the animals is the most valuable criterion. In our experience, the development of rachitic metaphyses is not an absolute indication of the severity of rickets because, after a certain point, with the slowing down of growth, the general symptoms of rickets may increase very decidedly without a proportionate increase in the width of the metaphyses. We have made it a point to take our rats for test when rickets has developed to such a degree that they become inactive and docile. They have then developed a swelling of the joints and a peculiar hopping and wabbling gait. At the next stage, when the enlargements have developed to a pronounced degree, the gait usually has become abnormal to such an extent that we speak of the animals as being stiff.

Series 2. Quartz Mercury Vapor Irradiation, 1926.

As our data on indirect activation of cow's milk by sunlight were not as definite as we had expected them to be from our experience with the use of artificially generated ultra-violet light on goats, we

were led to determine the effect of the stronger ultra-violet radiation on two of the cows, namely Cows 1 and 2.

Cow 1 was irradiated $\frac{1}{2}$ hour daily except Sunday with a new Cooper Hewitt poultry treater at a distance of 20 inches over the back. Milk was taken every other day beginning December 1, after the cow had been irradiated since November 6 for a total of 10 hours. Irradiation was continued in this manner until December 14, when the lamp was shifted to a position directly over the animal's head. On January 8, tests were again begun. Starting with January 20 the point of radiation was again shifted—this time being directed upon the udder from the rear with the tail tied over to one side. For this we used a new Hanovia Alpine Sun burner provided with a large reflector, at a distance of 2 feet.[1] Cow 2 was irradiated in the same manner as Cow 1 during the last period. Cow 1 produced 30 pounds of milk daily in November and 23 pounds in March. Cow 2 produced 30 pounds.

The milk from Cow 1 after the animal had been irradiated 24 days over the back, 25 days over the head, and finally 36 days on the udder, was found slightly curative of rickets when given in a dose of 8 cc. daily according to the previously described technique. The milk from Cow 2 was curative in 12 cc. doses after 36 days irradiation from the rear. These quantities were of the same order as those found necessary to produce a curative effect without irradiation.

Series 3, 1926.

The indefinite results obtained with Cows 1 to 6 led us later to resort to a different technique. Here we had at least two alternatives open. We could feed the milk over a longer period than 10 days to intensify the effect of small amounts and then carry out the silver nitrate test for gross comparisons, or we could feed milk together with Ration 2965 from the very beginning and then depend upon ash analysis of the bones for accurate quantitative comparisons. A third possibility, that of increasing the amount of milk fed with Ration 2965, was excluded because of failure to secure consumption of larger amounts than were previously

[1] The writers are indebted to the Hanovia Chemical and Manufacturing Company and the Cooper Hewitt Electric Company for the loan of these lamps.

administered with the basal ration continuously available. It was decided to use the second alternative. Accordingly milk from two cows was fed fresh before and after irradiation—each time to two litters of rats. As each litter contained six rats, it made it possible to administer the milk in 2, 4, 6, and 8 cc. doses and still leave one rat for control on the basal ration without milk and one rat for 4 cc. of milk directly irradiated. The first control would give us minimum calcification and the second maximum.

Cows 7 and 8 were both pure bred Ayrshires. Both were entirely white over the back and from the rear except Cow 7, which had a few red spots on the hind quarters. Cow 7 produced 20 pounds of milk daily and Cow 8 19 pounds. Cow 7 had been milked 3 months when the experiment was started and Cow 8 1 month.

Milk was given to the rats in the aforementioned quantities daily for 5 weeks. The cows were then irradiated daily from the rear at a distance of 2 feet, with a Hanovia Alpine Sun burner with an especially designed reflector to project the rays horizontally. Irradiation was carried out daily $\frac{1}{2}$ hour for the first 23 days. Then the exposure was increased to 1 hour daily and continued thus for 29 days, when the feeding of milk was discontinued. The cows therefore had received a total of 9 hours irradiation before the feeding of milk was started and 41 hours when the experiment was discontinued.

The 4 cc. of milk directly irradiated were prepared by irradiating 50 cc. in a new pie tin, measuring $7\frac{3}{4}$ inches in diameter, under a Cooper Hewitt quartz mercury vapor lamp of the BY type. This was run at a burner voltage of 50 with a current density of 4 amperes. Exposure was made at a distance of 20 inches for 30 minutes.

The results of these trials are shown in Table III. From the point of demonstrating a definite effect of irradiating the cows, the results are disappointing, because the rats receiving milk from the irradiated cows showed rather uniformly less calcification than those receiving the original milk. This does not look reasonable because a depression was hardly to be expected. On the other hand, any experimental error except for that of an artefact we believed to have been excluded because the controls from eight different litters receiving 4 cc. of milk which had been directly

irradiated checked fairly well, and these determinations were run at the same time as the others and the analyses were made simultaneously. The same is true of the control groups which did not receive any milk. That the results of analyses of the rats receiving the milk from irradiated cows were actually lowered we believed to have been caused by the fact that these rats were from different litters and were younger and on the average lighter than the others. They were respectively 23, 24, and 25 days old and weighed 48 to 57 gm. when put on experiment. The other rats were respectively 23, 27, 25, and 29 days old and weighed from 52 to 60 gm. at the same time. Of the controls not receiving any milk the oldest within this range gave the highest ash value and the youngest the

TABLE III.

Direct Irradiation of Cows and Antirachitic Value of Milk. Comparative Prophylaxis of Rickets in Rats from Different Litters.

Milk daily, cc.	0	2	4	6	8	
	Percentage of ash in femurs (average of 2 rats).					
Cow 7. Not irradiated	29.8*	34.6	38.2	45.2	50.8	
Irradiated		29.8	33.5	41.7	42.5	53.0*
" 8. Not irradiated	28.0*	36.9	37.4	40.2	45.4	
Irradiated		30.9	32.6	34.7	41.4	50.5*

* Average at four rats, Litters 3645 to 3755 and 3647 to 3757 inclusive.

lowest, which bears out our assumption that the difference in the age of these animals, small though it was, was a very important factor in determining the final ash content of their bones. This, however, we are certain is not the only factor because we have recently found that even rats taken from the same litters and kept on the same ration, at the end of 5 weeks feeding, as well as at the beginning, may show variation in the percentage of ash found in their bones. This demonstrates the necessity of devoting more attention to the standardization of rats for such experiments as these and the desirability of using rats from the same litters.

Series 4, 1926–27.

Beginning with October, 1926, there was in progress an experiment with three cows, Cows 2, 6, and 9, with which it was at-

tempted to determine the effect of ultra-violet radiation upon the calcium balance during liberal milk secretion. These cows were fed a ration composed of yellow corn 59, bran 25, oil meal 15, sodium chloride 1, 1 pound for every 3 to 4 pounds of milk produced, with silage 30, timothy hay 5, and alfalfa 5, and had already been fed this ration for a period of 2 weeks when the experiment was started. They had been confined indoors for a considerable period of time. The results on the calcium balance have been published (24).[2]

Inasmuch as our previous results with the technique employed, both curative and prophylactic, were equivocal in character, it was clearly evident that if better data were to be accumulated these should be obtained with a technique modified to increase the sensitivity as well as the accuracy of the determinations. The greatest difficulty hitherto experienced in accentuating the calcifying qualities of the milk was in getting the rats to consume a sufficiency of milk to give a pronounced curative test. This suggested that it might be advisable to restrict the intake of the basal ration to such a degree that the amount of milk consumed would be increased.

To determine the possibility of the execution of such a technique, two groups of rats, namely Lots 3936 and 3937, were fed as follows: Rats 6285 and 6288 received milk only, which was given them in 10 cc. quantities daily. Rats 6283, 6284, and 6287 were given 50 cc. daily. Rats 6282 and 6286 were given 20 cc. of milk, and when this was consumed 10 gm. of Ration 2965 were made available for their consumption. Usually 5 gm. of this were consumed. The results of these trials were as follows: The rats on 10 cc. of milk died in 6 to 10 days. The rats on 50 cc. did not consume all of it for the first few days but did consume it completely by the end of the 10 day period. Their rachitic metaphyses were completely healed. The rats on 20 cc. of milk with Ration 2965 had metaphyses which were almost but not quite healed.

The last results suggested to us that 20 cc. of milk daily were the approximate maximum which it would be desirable to give any rat for a 10 day period of a rachitic test. It also suggested to us that 0, 5, 10, 15, and 20 cc. of milk respectively might be suitable

[2] In this publication Cows 2, 6, and 9 were numbered 1 to 3 respectively.

TABLE IV.

Direct Irradiation of Cow and Antirachitic Value of Milk. Days of Exposure of Cow and Calcium Deposition in Curative Experiments with Rats.

	Milk fed.	Before irradiation of cow.						After irradiation of cow for:								
								2 days	2 days	4 days	4 days	8 days	8 days	14 days	14 days	28 days
Rats of Litter.....		3958	3964	3980	3984	3991	4005	3980	3982	3984	3987	3991	3997	4005	4007	4016
	cc.															
Cow 6.	0	–	–	0	0	0	0	0	–	0	0	0	–	0	–	–
	5	–	–	+	++	?	?	+	+	?	? to +	?	+	?	?	?
	10	++	++	++	++	++	+	++	++	++	+	++	++	+	+	++
	15	++	+++	+++	++	+++	+++	+++	++	–	++	+++	++	++	++	++
Rats of Litter.....		3956	3970		3985	3992	4003	3981		3985	3986	3992	3994	4003	4010	4020
Cow 9.	0	–	–		0	0	0	–		0	–	0	–	0	–	–
	5	?	?		+	?	?	+		+	?	?	–	?	+	?
	10	++	+		++	+	?	+++		+++	++	+	++	+++	++	++
	15	++	++		+++	++++	+++	+++		+++	+++++	++++	+++	+++	++	+++

0 = no test.
? = result in question.
– = negative result.
\+ = narrow line of calcium.
++ = medium line.
+++ = wide "
++++ = almost healed.
+++++ = healed.

levels at which milk should be fed, and that when these levels of milk were fed it might be advisable so to adjust the various amounts of Ration 2965 that the intake of total solids was approximately constant. As the intake with 20 cc. of milk and 5 gm. represented 7.6 gm. of total solids, a lower amount, namely 6 gm. of total solid, was assumed to be an acceptable level which would always be consumed.

Under this plan each rat after having developed the acceptable standard of severity of rickets would have its ration changed so that:

With	0 cc.	of	milk	it	would	be	given	6	gm.	of	Ration	2965
"	5 "	"	"	"	"	"	"	5.35	"	"	"	"
"	10 "	"	"	"	"	"	"	4.70	"	"	"	"
"	15 "	"	"	"	"	"	"	4.05	"	"	"	"
"	20 "	"	"	"	"	"	"	3.40	"	"	"	"

This method of feeding would not only induce the animal to consume a sufficient and constant amount of milk, but it would also increase the susceptibility of the rat to any antirachitic action. Milk itself carries so much phosphorus that when fed at high levels of intake it alone will tend to produce healing of the rickets induced by the basal ration in rats. Furthermore, as the intake of the basal ration is simultaneously reduced, diminution in the calcium carbonate intake results, which also facilitates healing. We therefore had reason to believe that the technique described above would lead to the production of accentuated effects induced by such antirachitic factors as might be present.

Experiments of the above nature were carried out with two of the cows, Cows 6 and 9. The results are shown in Table IV. They show that irradiation of both Cows 6 and 9 for a total of 28 hours distributed over as many days had no effect on the antirachitic value of the milk. Data secured on a 20 cc. intake of milk were not included in Table IV, because this produced normal calcification in all cases. It is to be added that Cows 6 and 9 were selected because their skins were the least pigmented and presumably would therefore be most affected by the process of irradiation. Cow 9 was almost entirely white over its back and Cow 6 was seven-eighths white, the rest being black.

We also used milk from these two cows for prophylactic test. Milk was collected on the last day of irradiation from both Cows 6 and 9 and was fed to three rats in each of three litters and compared directly with milk from the pre-irradiation period in the same litters. Both samples were sterilized at 15 pounds pressure for 30 minutes. They were fed at an intake of 4 cc. per rat daily. This amount of milk was selected because in the experiments of Cows 7 and 8 (Table III), it was seen that with the larger amounts of milk there was a distinct tendency to produce normal calcification. With smaller amounts, on the other hand, calcification was insufficient.

The results of these experiments, which were continued for a period of 5 weeks, were obtained in terms of per cent of ash in the alcohol-extracted dried humeri and femurs, as well as in terms of the comparative widths of the rachitic metaphyses of the distal ends of the radii and ulnæ as revealed by silver nitrate staining. These results are brought together in Table V.

Apparently the results here are slightly different than those of the earlier determinations, because more intense treatment with ultra-violet light apparently induced a slight increase in the calcifying properties of the milk; but the results are not decisive because of variations with individual animals. Comparison of the results with the milk of the pre-irradiation period with the milk of the irradiation period for Cow 9 shows that for the humeri the ash content is as 41.33 per cent compared with 43.02, a difference of 1.69, and for the femurs the ash content is 37.19 compared with 39.52 per cent, a difference of 2.33. With the humeri, the values with milk from Cow 6 are 40.92 compared with 42.17 per cent, a difference of 1.25, and the femurs 36.78 compared with 37.47 per cent, or a difference of 0.69. When it is noted that in the same groups we obtained with milk from Cow 9 differences of 10.65 per cent between individual rats, these values do not look so significant because in the average for the group this would produce a variation of 1.18 per cent. With milk from Cow 6 the largest variation is 8.19 per cent, which likewise would change the average by 0.91 per cent. The observed effect cannot be taken as an artefact induced by sterilization because both milks were sterilized. However, there is not excluded the possibility that the difference may have been caused by keeping the pre-irradiation milk until the irradia-

TABLE V.

Direct Irradiation of Cow and Antirachitic Value of Milk. Comparative Prophylaxis of Rickets in Rats from Same Litters with 4 Cc. of Milk.

	Rat litter No.	Rat No.	Treatment of cow.	Initial weight of rat.	Final weight of rat.	Ash in humeri.	Ash in femurs.	Width of metaphyses.
				gm.	*gm.*	*per cent*	*per cent*	
Cow 9.	4062	6949	Not irradiated.	50	93	44.51	40.41	Medium.
		4950	" "	54	128	34.77	31.49	Very wide.
		6951	" "	52	106	40.62	37.40	Medium.
				52	**109**	**39.97**	**36.43**	
		6952	Irradiated 4 wks.	54	98	43.54	40.88	Narrow.
		6953	" 4 "	51	107	43.52	41.14	Medium.
		6954	" 4 "	51	106	40.74	37.59	"
				52	**103**	**42.60**	**39.87**	
	4065	6987	Not irradiated.	52	123	41.44	38.57	Wide.
		6968	" "	48	114	39.76	35.83	"
		6969	" "	50	110	44.40	39.59	Narrow.
				50	**115**	**41.87**	**38.00**	
		6970	Irradiated 4 wks.	48	95	48.76	45.95	Narrow.
		6971	" 4 "	52	122	43.66	39.47	Medium.
		6972	" 4 "	45	118	38.95	35.30	Wide.
				48	**111**	**43.79**	**40.24**	
	4067	6979	Not irradiated.	50	104	42.58	36.91	Medium.
		6980	" "	48	100	42.72	38.17	"
		6981	" "	56	137	41.14	36.34	Wide.
				51	**114**	**42.15**	**37.14**	
		6982	Irradiated 4 wks.	50	120	42.24	37.43	Wide.
		6983	" 4 "	55	112	45.41	41.96	"
		6984	" 4 "	53	117	40.39	36.00	"
				52	**116**	**42.68**	**38.46**	
Cow 6.	4068	6985	Not irradiated.	50	108	40.42	35.52	Narrow.
		6986	" "	45	80	45.48	40.47	"
		6987	" "	50	103	41.12	36.20	"
				48	**99**	**42.34**	**37.40**	
		6988	Irradiated 4 wks.	50	102	46.04	39.83	Medium.
		6989	" 4 "	50	102	39.38	33.98	"
		6990	" 4 "	50	108	37.85	30.87	Wide.
				50	**104**	**41.09**	**34.89**	

Figures set in bold-faced type denote averages.

TABLE V—*Concluded.*

	Rat litter No.	Rat No.	Treatment of cow.	Initial weight of rat.	Final weight of rat.	Ash in humeri.	Ash in femurs.	Width of metaphyses.
				gm.	*gm.*	*per cent*	*per cent*	
Cow 6—continued.	4071	7000	Not irradiated.	48	96	43.15	39.73	Narrow.
		7001	" "	50	100	44.72	39.31	Medium.
		7002	" "	47	78	40.82	36.81	Narrow.
				48	**91**	**42.90**	**38.62**	
		7003	Irradiated 4 wks.	50	127	41.99	37.62	Narrow.
		7004	" 4 "	49	106	44.64	41.36	"
		7005	" 4 "	45	96	44.82	41.82	"
				48	**109**	**43.82**	**40.27**	
	4078	7042	Not irradiated.	48	96	38.12	34.47	Narrow.
		7043	" "	50	104	37.40	34.11	Wide.
		7044	" "	50	102	37.03	34.36	"
				49	**100**	**37.52**	**34.31**	
		7045	Irradiated 4 wks.	51	103	43.15	37.50	Narrow.
		7046	" 4 "	50	100	42.44	38.79	"
		7047	" 4 "	48	99	39.17	35.50	"
				49	**100**	**41.59**	**37.26**	

tion milk was available. This, however, we do not believe to be probable. It therefore appears that a slight increase in antirachitic value in the milk of cows was probably induced by the intensive irradiation with ultra-violet light in these experiments.

Series 5, 1927–28.

Our experiments were continued with refined technique in the winter of 1927–28 with two cows, Cows 10 and 3, both Holsteins. Cow 10 was selected because it had not been exposed to sunshine since February of the preceding year and because it was more white than black. Cow 3, however, had been exposed to sunshine during the summer with the University herd; this cow was approximately one-half black and one-half white.

The pre-irradiation period during which milk was collected for determinations of antirachitic value was begun on December 1

with Cow 10 and on December 13 with Cow 3. Irradiation was begun on January 17 and continued to March 9 with quartz mercury vapor lamps of the Cooper Hewitt and Luxor alternating current types. They were run on our alternating current of 110 volts. Exposures were made for 1 hour daily with the arc suspended 24 inches above the backs of the cows, one lamp being used for each cow.

The cows were milked twice daily and milk records with analyses of milk for butter fat kept throughout the experiment. Milk was kept daily for tests of antirachitic potency, and in addition, butter was prepared in quantity from the milk of each cow during the pre-irradiation period as well as during irradiation for a further check on its potency.

The tests for antirachitic potency were carried out with rats ranging in weight from 54 to 66 gm. at an age of 23 to 29 days. They were distributed among the different groups so that each litter was represented in each of the groups and each group contained the same number of individuals from each litter.

In the many hundreds of determinations which we have made of the comparative ash content of the bones of rats, we have found that in general the variations among individuals from one litter are far less than those from different litters. We have therefore been impressed with the desirability of using rats from the same litter. Obviously in these experiments where the samples of milk from the pre-irradiation period could not be collected at the same time as from the irradiation period, the feeding of the samples to different litters was unavoidable unless the samples were preserved during the interim. In order to equalize the effect of the variations, allowing us at the same time to collect data on the effect of keeping milk from one period to the other, we fed our milk samples in the raw condition, sterilized for 30 minutes, and for 120 minutes. Sterilization was effected by heating in an autoclave at 15 pounds pressure. During the irradiation period the milk was fed raw and heated for 30 minutes and both samples compared directly in the same series with milk from the pre-irradiation period, likewise heated for 30 minutes. This arrangement allowed us to obtain data on the effect of sterilization and made it possible to compare the calcifying potency of the milk from both periods between differ-

ent litters and in the same litters. In none of these experiments, however, were all the factors taken care of, but we believe that by attacking the problem from these various angles our data would give us as accurate comparisons as our facilities allowed.

TABLE VI.

Percentage Ash in Femurs of Rats Receiving 4 Cc. of Milk Daily from Non-Irradiated As Contrasted with Irradiated Cows.

	Rat No.	Pre-irradiation period.					
		Raw milk.		Milk autoclaved 30 min.		Milk autoclaved 120 min.	
Cow 10.	1–6	36.64	41.03	36.55	35.47	34.23	34.18
	7–12	31.73	36.96	36.56	33.23	28.74	31.32
	13–18	33.57	35.20	30.98	29.28	30.34	32.89
Average		35.86		33.68		31.97	
Cow 3.	37–42	37.69	32.57	32.19	33.36	34.68	34.77
	43–48	40.77	39.10	36.92	33.50	33.60	33.45
	49–54	36.52	38.86	36.59	34.64	42.50	35.52
Average		37.59		34.53		35.77	
	Rat No.	Irradiation period.					
		Raw milk.		Milk autoclaved 30 min.		Milk from non-irradiation period autoclaved 30 min.	
Cow 10.	55–60	40.55	41.39	41.53	40.75	40.50	40.34
	61–66	32.18	34.00	31.78	36.64	36.99	34.37
	67–72	43.03	37.39	37.39	36.99	37.17	36.17
Average		37.13		37.51		37.59	
Cow 3.	73–78	35.09	40.44	33.62	37.27	36.63	31.32
	79–84	35.42	30.33	30.30	32.18	27.19	34.48
	85–90	32.80	34.55	33.97	38.32	35.61	37.16
Average		34.77		34.28		33.73	

Data on the comparative antirachitic potency of the milk from the two periods are presented in Table VI. The results on the effect of heat are not clean cut. During the irradiation period 30 minutes heating revealed no effect, but during the pre-irradiation period the ash content of the bones was reduced approximately 2.4

per cent by such treatment. Variations in the ash content of bones of rats taken from different litters being disregarded, it is seen that irradiation of the cow had no effect upon the antirachitic potency of the milk. This is supported by the fact that when we compared sterilized milk from both the pre-irradiation period and the irradiation period in the same litter we got practically identical values for the calcification effected.

TABLE VII.

Sterilization and Stability of Vitamin D in Milk Prepared from Irradiated Skimmed Milk Powder.

Rat No.	Milk addition to Ration 2965.	Ash in femurs.				Average.	Width of metaphyses.	Enlargement of costochondral junctions.
		per cent	*per cent*	*per cent*	*per cent*	*per cent*		
65–68	2 cc. daily, not sterilized.	42.55	46.07	42.44	46.13	44.30	Narrow to medium.	Slight to medium.
53–56	2 cc. daily, sterilized.	38.40	38.61	42.27	41.71	40.25	Medium with cupping.	Medium.
69–72	4 cc. daily, not sterilized.	49.50	51.52	49.61	48.30	49.74	Narrow with cupping (normal epiphyseal line, Rat 70).	Slight (almost normal, Rat 70).
57–60	4 cc. daily, sterilized.	43.52	47.87	45.90	48.30	46.40	Medium.	Slight.

Though the reduction in calcification by the heated milk as compared with the unheated was of a rather low order, yet we felt it to be of sufficient importance to warrant further investigation. The literature contains numerous references dating many years back purporting to show that heated milk is less efficient in covering the calcium requirements of the infant than raw milk. We took especial precautions when feeding our heated milk to shake it up thoroughly and to remove samples as representative as possible from the containers. We believed that we succeeded in securing these although, of course, we are not absolutely certain that the

TABLE VIII.

Effect of Irradiation of Cow on Antirachitic Potency of Butter Fat.

Rat No.	160 mg. butter fat added to Ration 2965.	Per cent ash in femurs.				Grand average.	Width of metaphyses.	Enlargement of costochondral junctions.
360–363	From Cow 3, pre-irradiation period.	34.68	28.70	38.32	30.57		Wide rachitic.	Pronounced; extreme angulation in Rats 361, 363.
		34.21	28.89	37.27	29.82			
		34.4[illegible]	28.79	37.79	30.19	32.79		
364–367	From Cow 3, irradiation period.	27.74	28.20	37.33	31.62		Wide rachitic. Slight calcification in Rat 366.	Pronounced; extreme angulation. Medium enlargement, Rat 366.
		26.33	27.23	37.03	30.44			
		27.03	27.72	37.18	31.03	30.73		
380–383	From Cow 10, pre-irradiation period.	29.98	25.90	32.07	28.19		Wide rachitic.	Pronounced; extreme angulation.
		31.41	26.95	32.36	28.54			
		30.70	26.43	32.22	28.37	29.43		
368–371	From Cow 10, irradiation period.	26.78	36.36	30.77	35.48		Wide rachitic. Medium to narrow in Rat 369.	Pronounced; extreme angulation. Medium enlargement, Rat 369.
		26.90	36.36	30.77	35.48			
		26.84	36.36	30.77	35.48	32.36		
372–375	Sample of Apr., 1928, University creamery.	30.20	40.88	31.55	36.63		Wide rachitic in Rats 372, 374. Narrow to medium in Rats 373, 375.	Pronounced; extreme angulation, Rats 372, 374. Medium enlargement, Rat 373.
		29.90	41.14	30.74	35.87			
		30.05	41.01	31.15	36.25	34.62		
376–379	Sample of June, 1927, University creamery.	47.69	39.47	44.81	43.29		Narrow.	Medium.
		48.22	40.93	44.09	42.63			
		47.96	40.20	44.45	42.96	43.89		

rats may not have left very small amounts of the calcium salts in their dishes even though, as far as we could tell, the milk was completely consumed.

In Table VII are presented further data on the comparative calcifying effect of sterilized and unsterilized milk, obtained in this case by feeding solutions of skimmed milk powder. Milk powder was used because it enabled us to feed fresh solutions, uniform in composition, which solutions we could sterilize as desired. The powder was irradiated with an Alpine Sun lamp, run at low intensity for 30 minutes with stirring after the first 15 minutes of exposure. The exposure was made at a distance of 18 inches with 57.5 gm. at a time, sifted into a galvanized iron pan 2 feet square. For feeding, the powder was taken in solution, 9.5 gm. in 100 cc. of distilled water. Sterilization was effected in small Erlenmeyer flasks at 15 pounds pressure for 30 minutes. 2 to 6 cc. were fed daily. Table VIII, which shows the results obtained at the 2 and 4 cc. levels of intake, indicates that the antirachitic potency of the milk was markedly reduced, but was far from being destroyed. Natural milk at a 4 cc. level of intake produces bone of 31 to 37 per cent ash. Obviously this irradiated milk, producing bone of 48 to 49 per cent ash, was far more potent and therefore in better position to reveal the lability of the antirachitic factor. These results reveal that sterilization does bring about a decrease in antirachitic potency.

Fortunately, in our experiments, milk from our non-irradiation periods was always collected before direct irradiation of the cows and therefore had not only been sterilized but had been stored as well, though at ice box temperatures, before feeding. This would naturally have increased the difference in calcification. The fact that little if any difference in calcification was observed makes it reasonably certain that irradiation with intense ultra-violet light has no effect.

Table VIII presents the results on butter fat. It verifies the results obtained with the milk. 160 mg. of butter fat were fed daily from both cows, and the results from these compared with the April, 1928, butter fat, as well as with June, 1927, butter fat. The technique was the same as with milk. Ration 2965 was used as the basal. Both the latter samples were obtained from the general run of the University creamery.

It will be noted that there is no significant variation in the calcifying properties of the butter fat obtained from the cows subjected to irradiation, nor does this butter fat differ markedly from the April butter fat obtained from the University creamery, but all of these samples are markedly lower than the butter fat collected in June while the cows were out on pasture.

These results, we believe, prove conclusively that the antirachitic potency of milk or of butter fat obtained therefrom is dependent primarily upon other factors, possibly the feed consumed by the cow, and only secondarily, if at all, upon ultra-violet rays acting upon the animal's body.

Effect of Irradiation on Milk Production.

In view of the constitutional effect of ultra-violet light, the possibility of its affecting milk secretion in other ways than improvement in quality is worthy of consideration. Iguchi and Mitamura (25) concluded that irradiation of a cow's udder increased milk volume 4.77 per cent and butter fat 8.73 per cent. Scolte and Wiener (26) found increased milk secretion with twenty mothers whose breasts were irradiated to an erythremic state.

Our data on milk and butter fat production which were obtained incidentally with our other observations do not reveal anything new. Cow 10 fell off in daily milk production from 26.9 to 24.7 pounds and Cow 3 from 19.2 to 17.1 pounds during the irradiation, as compared with the pre-irradiation period. One butter fat test changed from 3.31 to 3.68 per cent with Cow 10 and remained constant at 3.40 per cent with Cow 3. These fall well within the range of the usual variations which occur with lapse of time.

It, however, is to be pointed out that in these experiments the udders were not irradiated directly as in the experiments of Iguchi and Mitamura and Scolte and Wiener just cited. In our Series 3, Cows 7 and 8 were irradiated on their udders 30 to 60 minutes daily. The weekly averages for 4 weeks immediately preceding irradiation compared with the values 4 weeks after irradiation show that Cow 7 produced 181 pounds of milk before and 153 after; Cow 8, 166 pounds before and 140 after. For butter fat Cow 7 produced 3.7 per cent before and 4 per cent after irradiation, and Cow 8, 3 per cent before, 3.8 per cent after; total pounds of butter fat: Cow 7 6.7 pounds before, 6.1 after; Cow 8 6.2 pounds

before, 5.3 after. These figures do not indicate any effect in one direction or another.

SUMMARY.

Daily exposure of cows to sunlight or artificially generated ultraviolet radiations has little if any effect on the antirachitic potency of milk. These experiments were carried out with Ayrshire and Holstein cows with coats for the most part unpigmented and with the radiations falling on the head, back, or udders, the latter being almost free from hair. The radiation period in some cases was continued for an hour daily at 20 to 30 inches with Cooper Hewitt or Alpine Sun lamps. Rats were used as the test animals for both prophylactic and curative technique. The results stand in marked contrast to our earlier observations with goats. No improvement in milk or butter fat secretion was observed. The well recognized superior quality of summer-produced milk and butter fat must therefore have its primary origin in other factors than sunlight acting directly upon the cow.

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FAT-SOLUBLE VITAMINS.

XXIX. IS ANTIRACHITIC ACTIVATION INDUCED BY ULTRA-VIOLET RADIATIONS A PANACEA FOR NEGATIVE CALCIUM BALANCES?*

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The nature of the data obtained with cows showing that ultra-violet irradiation has but very little if any effect upon the anti-rachitic value of milk (1) led us to reexamine our results with goats although we had no specific reason to doubt the reliability of our previous conclusions (2). In most respects the technique employed in these experiments was the same as that used by us with cows. We did, however, make one modification because we wanted to avoid the possibility of the goat obtaining activated compounds from its body by licking. To achieve this we confined the goat in a stanchion attached to a metabolism cage as shown in Fig. 1.

Goat 1, a white goat weighing approximately 68 pounds after having been confined in the barn all winter, was brought to the laboratory in an early stage of lactation on April 22 for this experiment. The goat was confined in the metabolism cage to make it possible to collect excreta and yet keep the animal fairly comfortable. Goats are usually very adaptable to new and strange conditions and we soon found that Goat 1 would extend its fore legs into the manger to lick them. To prevent this we suspended a light board from its neck by means of a strap. This board moved up and down in a groove in the side of the stanchion and accomplished our purpose without discommoding the animal in the least.

As a ration there was provided a grain mixture consisting of

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yellow corn 59, wheat bran 25, linseed oil meal 15, and sodium chloride 1. This was fed with alfalfa hay in the proportion of 2 parts of grain to 1 of hay, giving a total of 4200 gm. of grain and 2100 gm. of hay per week. For the last 4 weeks of the experiment our original supply of grain was exhausted and it was necessary to provide a new mixture. This was compounded from similar ingredients but differed slightly in its mineral content.

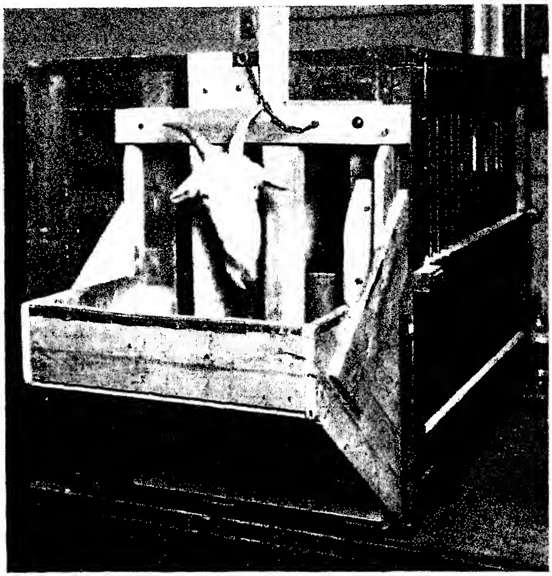

FIG. 1. Metabolism cage provided with a special stanchion to prevent the goat from licking its body.

Beginning May 22 the goat was irradiated 30 minutes daily with a new Cooper Hewitt lamp, type BY, run on a direct current of 50 volts and 4 amperes. For this the goat was transferred to a special stanchion, as shown in Fig. 2. Inasmuch as this goat was long haired, the precaution of clipping was taken at the beginning of the experiment on April 29. This did not denude the skin of hair, but left a very fair covering probably not so very different from that of a cow. For the most part the burner was distant only 6 to 8 inches from the goat's body. The head and ears were completely protected from the direct rays of light. They were, however, exposed to such rays as were reflected from the walls of the room.

Effect of Irradiation on the Antirachitic Value of Milk.

Milk was drawn daily, morning and evening, measured, and samples kept for analysis and determination of the antirachitic value. For the pre-irradiation trials for rats of Lots 3773 and 3774, it was used fresh, but later, to make it possible to obtain direct comparison at different times and also when using rats from the same litters, the milk was sterilized for 30 minutes at 15 pounds pressure and then kept in a refrigerator until fed.

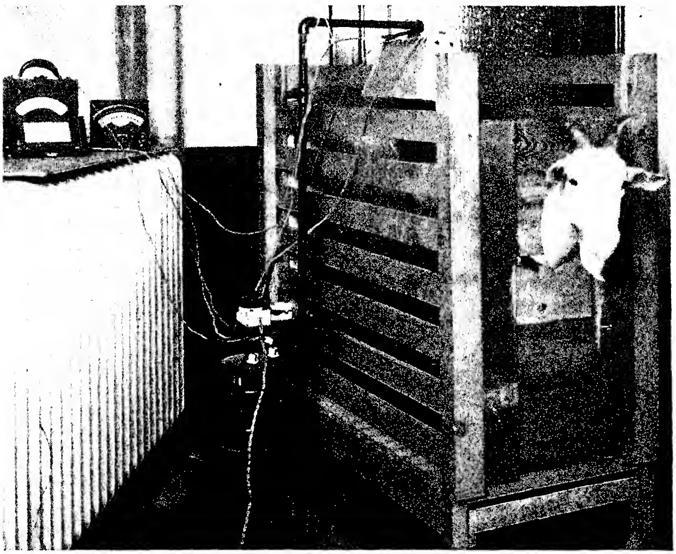

FIG. 2. Irradiation cage and quartz mercury vapor lamp.

The antirachitic tests were made with rats, both the curative and prophylactic types of experiments being employed. In the first set of curative experiments the milk was fed at 2, 4, 6, and 8 cc. levels of intake with one control rat not receiving any milk and another receiving 1 cc. of milk in which maximum antirachitic activity had been induced by direct irradiation with a quartz mercury vapor burner as previously described (2). In a second set of curative experiments milk taken at different times (early and very late) during the irradiation process was fed to rats in the same litters, at levels of 2, 4, and 6 cc. per rat daily.

TABLE I.

Antirachitic Activation of Milk by Irradiation of Goat.

Rats of Lot No.	Before irradiation of goat, 10-1 days.			After irradiation of goat. 1 day.	2 days.	4 days.	8 days.		31 days.		
	3804	3773	3774	3807	3808	3809	3811	3812	3844	3846	3842
cc.											
Non-irradiated milk.											
0	−	0	0	−	−	−	−	−	−	0	−
2	−	−	−	−	−	−	+	−	+	−	−
4	?	?	−	−	−	?	+	+	++	+	++
6	+	+	+	+	?	++	+++	++	+++	++	++
8	+	++	++	++	++	+++	+++	+++	+++	+++	+++
Irradiated milk.											
1	+++	+++	+++	+++	+++	+++		+++			

0 = no test.
− = negative result.
? = doubtful "

\+ = narrow line of calcium.
++ = medium " " "
+++ = wide " " "

The rats used for these trials were made rachitic by feeding our Ration 2965 (2) for a period of 21 to 24 days. Just as soon as these animals became inactive and began to walk with a distinct sham-

TABLE II.

Comparative Antirachitic Value of Goat Milk at Beginning and Close of Irradiation, with Comparison Made in Same Litter of Rats at Each Level of Milk Intake.

Rats of Litter No.	Volume of milk fed.	Interval after irradiation.			
		1 day.	2 days.	28 days.	
	cc.				
3848	2	–	–	–	–
3849	4	–	?	++	++
3850	6	?	+	+++	+++

TABLE III.

Antirachitic Activation of Milk by Irradiation of Goat.

Volume of milk fed.	Treatment of goat.	Per cent of ash.					
		Rats of Litter 3857.		Rats of Litter 3858.		Rats of Litter 3859.	
		Humerus.	Femur.	Humerus.	Femur.	Humerus.	Femur.
cc.							
2	Before irradiation.	34.06	32.07	37.73	31.47	39.68	35.84
	After "	47.56	46.02	47.48	42.74	36.16*	33.23*
4	Before irradiation.	37.66	35.44	36.75	33.62	41.74	38.21
	After "	48.81	48.66	47.46	44.05	47.26	44.70
8	Before irradiation.	46.09	46.54	43.46	37.41	43.12	40.42
	After "	39.16†	36.58†	57.06	56.20	54.38	56.03

* For these unusually low results we have no explanation.

† Rat lost weight.

bling and wabbling gait they were used for the experiment. This can be ascertained with a fair degree of uniformity by daily observation of the animals and constitutes a very important feature in the selection of the animals. When ready for the experiment our

animals weighed on an average for the groups between 80 and 96 gm. After having received the daily additions of milk to their diet, which were made during 10 days, they weighed on the average 90 to 113 gm. Consumption records were taken daily during the experimental period and if for any reason failure of appetite occurred, the test was no longer considered of any value and was discontinued. With the conclusion of the feeding period the distal ends of the radii and ulnæ were examined for Ca deposits by the Johns Hopkins line test (3).

The prophylactic experiments were carried out by feeding milk from the pre-irradiation period as well as the irradiation period—the samples being fed at 2, 4, and 8 cc. levels of intake per rat daily within individual litters. This was done in order to eliminate variation in individual animals as much as possible. The rats weighed 56 to 64 gm. at the time the experiment was started when they were 25 to 30 days old. They were given the quantities of milk mentioned above in addition to Ration 2965 which was fed *ad libitum* for a period of 5 weeks, at the end of which time they weighed from 105 to 172 gm., averaging 128 gm. After being killed with ether, the humeri and femurs were dissected out, extracted with fresh changes of hot alcohol in a Soxhlet apparatus for a few days, then dried, and ashed in an electric muffle furnace.

The results of the above experiments are shown in Tables I to III. They bear out our previous conclusions that direct irradiation of the goat is able to increase the antirachitic properties of the milk secreted, and in these experiments, just as in the previous ones, we obtained undisputed evidence that this increase in activity took place after 4 days of irradiation when the animal had been exposed to ultra-violet light for a total period of 2 hours. This, of course, is not an absolute value; it undoubtedly varies with the duration of each exposure and its intensity but unquestionably a certain latent period preceding the effect on the milk is necessary as it must take some time for the activated compounds to be reabsorbed and transported into the mammary gland. Inasmuch as the utmost precautions were taken to keep the animal from licking itself or consuming any activated materials either in its ration or foreign to it the possibility of the observed effect being achieved in any other manner than through absorption from the skin was definitely excluded. The goat accordingly appeared to

be more reactive to ultra-violet radiations than cows. Whether or not this is dependent upon mere difference in the relation of total body surface to amount of milk secreted or whether there are physiological differences in activity of the skin and in the translocation of the activated compounds remains to be determined.

Effect of Irradiation upon Calcium Balance.

It has already been determined in this laboratory that irradiation of a goat will change a negative calcium balance into a positive one even though the level of calcium intake is kept the same (4). It was accordingly of interest to determine with Goat 1 whether there was any distinct time relation between the first observation of increase in the antirachitic properties of the milk and the change in calcium retention.

To determine this, careful record of feed consumption was kept, and feces, urine, and milk were collected quantitatively and all analyzed for calcium. For this, suitable proportions of the feed, feces, and milk were ashed and calcium determined by the McCrudden method. In the urine, however, the calcium was precipitated directly.

The amounts of feces, urine, and milk and their percentage content of calcium oxide week by week are shown in Table IV. Table V gives the total amounts of calcium oxide in feces, urine, and milk together with the figures for the calcium oxide intake, with the full calcium oxide loss and the calcium oxide balances week by week.

As the goat was exposed to ultra-violet rays day by day, it was observed that after the first few weeks the animal became very restless, frequently bleating, and at times throwing its weight from side to side and from foot to foot. Especially did it do this while being irradiated. Inspection revealed a slight epidermal roughness, though blistering and erythema were never observed. It was also noted that as this restlessness developed the goat no longer consumed its ration with the usual gusto, and on July 2 refused its ration entirely, thereby terminating the experiment.

The results on the calcium balance showed that, while possibly the retention of calcium was slightly greater during the early period of ultra-violet treatment, later on the losses of calcium oxide increased gradually from a few gm. to 10 gm. weekly. That anti-

TABLE IV.

Detailed Data on Calcium Output of Goat 1.

Period.	Weight of feces.	CaO in feces.	Weight of milk.	CaO in milk.	Volume of urine.	CaO in urine.
	gm.	*per cent*	*gm.*	*per cent*	*cc.*	*per cent*
Pre-irradiation.						
Apr. 29–May 6	1605	2.16	7170	0.192	2480	0.0213
May 6–13	1690	2.15	6142	0.179	2150	0.0233
" 13–20	1613	2.19	5375	0.190	2770	0.0235
" 20–27*	1620	2.44	5011	0.191	1560	0.0338
Irradiation.						
May 27–June 3	1557	2.24	4925	0.174	1760	0.0332
June 3–10	1685	2.15	4800	0.177	1840	0.0351
" 10–17	1632	2.54	4377	0.200	1930	0.0380
" 17–24	1671	2.51	4785	0.176	1450	0.0480
" 24–July 1	1502	2.88	4490	0.208	2100	0.0491

* Irradiation was started May 22.

TABLE V.

Calcium Balance of Goat 1.

Period.	CaO in feces.	CaO in milk.	CaO in urine.	Total CaO lost.	CaO intake.	CaO balance.
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Pre-irradiation.						
Apr. 29–May 6	34.66	13.76	0.62	49.04	45.36	−3.68
May 6–13	36.33	10.99	0.50	47.82	45.36	−2.46
" 13–20	35.32	10.21	0.65	46.18	45.36	−0.82
" 20–27*	39.52	9.57	0.57	49.66	45.36	−4.30
Irradiation.						
May 27–June 3	34.87	8.56	0.58	44.01	45.36	+1.35
June 3–10	36.22	8.49	0.64	45.35	43.26	−2.09
" 10–17	42.45	8.75	0.73	51.93	43.26	−8.67
" 17–24	41.94	8.42	0.69	51.05	43.26	−7.79
" 24–July 1	43.25	9.33	1.03	53.61	43.26	−10.35

* Irradiation was started May 22.

rachitic activation of organic substance was actually taking place was, of course, indisputably proved by our rat feeding experiments which showed an increase in the antirachitic potency of the milk.

Some years ago it was shown at this station (5) as well as at the Geneva station (6) that a negative calcium balance can be brought about when a lactating animal is not provided with sufficient phosphorus in the ration. Under such conditions with the strain of milk production, phosphorus will be withdrawn from the skeleton and an equivalent amount of calcium excreted through the feces as a waste product.

Inspection of the intake of phosphorus of Goat 1 as calculated from average phosphorus values of feed and milk showed that the intake was in the neighborhood of 70 gm. weekly and that the loss through the milk was 12 to 13 gm. weekly. It was evident from this that the observed calcium losses could not have had their origin in the reaction described above.

Meigs, Blatherwick, and Cary in 1919 (7), as a result of observations on cows and a critical analysis of data on the loss of calcium and phosphorus as recorded in the literature, came to the conclusion that, "The separate collection of urine and feces by attendants, as practiced in balance experiments with cows, produces a nervous disturbance in the animals which interferes markedly with the assimilation of calcium, and, to a less degree, with that of nitrogen and phosphorus." In the light of our observation that Goat 1 became exceedingly restless with the prolongation of the experiment, it is possible that the loss of calcium observed was induced by nervous disturbances as suggested by Meigs, Blatherwick, and Cary. As to what the specific reaction consisted of—whether it was the prolonged close confinement in the cage or irritation induced by excessive irradiation—we are not in a position to say.

A recognition of the fact that the excretion of calcium similar to the secretion of digestive juices may be partly under the control of the nervous system, we believe to be very important not only from the standpoint of suggesting the precautions which must be adopted in experiments of this nature but also from the standpoint of the therapeutic treatment of rickets. Leites (8) in 1925 showed that section of a vagus nerve in dogs led to a reduction in the K:Ca coefficient of serum with a later return to normal or even

hypernormal. The Ca in the serum was increased. Section of abdominal sympathetic nerves led to a decrease. Hess, Berg, and Sherman (9) confirmed the results of Leites on the splanchnic nerves and found that section of the cord caused an increase. It is occasionally reported that a rachitic condition is unilateral or, if of the usual type, nevertheless has failed to yield to treatment with ultra-violet light or cod liver oil. We believe it to be entirely possible that in such cases failure may have been due to intercurrent nervous disturbances such as those elicited by extreme deformities, infections, or digestive troubles, though, of course with cod liver oil, failure of absorption of vitamin D is a possibility when digestive disturbances obtain. Fortunately rickets represents a disease in which, during the early stages, the infant becomes lethargic rather than irritable so that under ordinary conditions the above reactions are usually not encountered.

Another possible explanation merits serious consideration. It is now a well established fact that a hypervitaminosis, due to excessive ingestion of irradiated erogsterol, causes a mobilization of calcium which leads to the deposition of excessive Ca in the heart, lungs, and kidneys with an increase in the Ca of the blood stream and urine. It is possible, though not probable, that Goat 1 was hypervitaminotic. To accept such a theory as a probability one would have to assume the presence of an unusual quantity of an activatable substance in the goat's body or a high sensitivity. Neither of these is acceptable. The ration of Goat 1 was not inordinately high in activatable substances and as goats are well known to thrive in sunny climates it appears doubtful that they would be lacking in an adequate protective mechanism against excessive ultra-violet radiations. We are inclined to entertain the possibility that reaction of Goat 1 to long continued irradiation under confinement was determined by a complex mechanism in which vitamin D, the nervous system, and the parathyroids were probably all factors. The elucidation of their rôle individually and collectively offers many opportunities for further research which is now in progress.

CONCLUSIONS.

In an experiment in which the utmost precautions to exclude the consumption of activated compounds were taken, it was found

that direct exposure of a goat to the radiations of a quartz mercury vapor lamp increased the antirachitic value of its milk very decidedly. This was in direct contrast to our experience with cows. Yet in spite of this it was observed that ultimately the goat showed a very decided negative calcium balance although originally with the beginning of irradiation a slight improvement in calcium retention resulted. In view of a simultaneous increase in the restlessness of the animal with the incidence of a pronounced negative calcium balance, it appears that the excretory elimination of calcium may be controlled neurologically as well as by the antirachitic factor. Whether or not this is effected directly or through some other mechanism, as for example the parathyroid glands, is not known. Antirachitic activation therefore cannot be considered a panacea for a disturbed calcium metabolism.

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A STUDY OF THE ANTIMONY TRICHLORIDE COLOR REACTION FOR VITAMIN A.

II. THE DILUTION CURVE OF COD LIVER OIL WITH ANTIMONY TRICHLORIDE REAGENT.

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In a previous paper of this series (1) we have shown that while the dilution curve for cod liver oil with antimony trichloride is not a linear function, a comparison can be made between the colorimetric assay of vitamin A in certain fish oils, with a chloroform solution of antimony trichloride, and the biological assay upon the same oils, when certain precautions are taken. In a second paper (2) it was shown that the color produced with the unsaponifiable substance from cod liver oil with the antimony trichloride reagent is a linear function of the amount of unsaponifiable substance used, below the maximum color which can be produced with a given concentration of antimony trichloride; that deviation of the dilution curve with oils from a linear function at relatively low color values is due to some interfering substance present in the oil (possibly due to the formation of a more stable compound between antimony trichloride and some substance or substances in the oil than is formed between antimony trichloride and the blue-producing substance of cod liver oil).

In this paper dilution curves of several fish liver oils are given and a correlation shown between the colorimetric and biological assay of the oils.

EXPERIMENTAL.

Dilution curves of several fish liver oils were made by determining the color produced by solutions of various concentrations of oil in chloroform. In each color determination 0.3 cc. of the chloroform solution of oil was used with 3.0 cc. of the chloroform

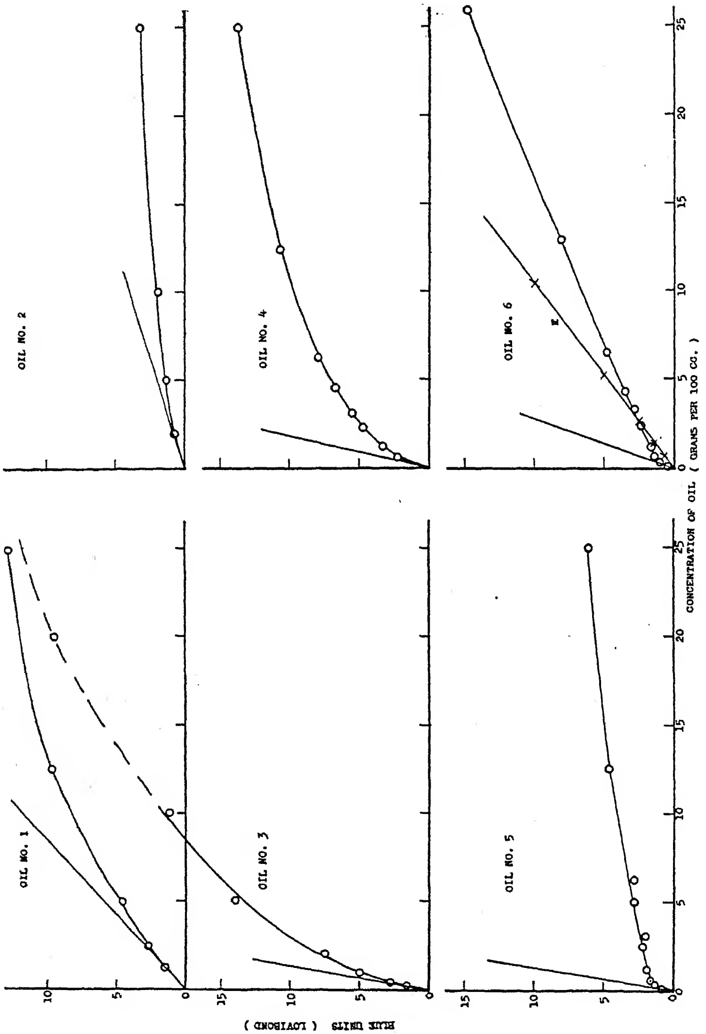

FIG. 1. Dilution curves of six fish liver oils. Intensity of blue color produced plotted against the concentration of the chloroform solution of oil used in making the test.

solution of antimony trichloride, saturated at the temperature of ice water, according to the technique outlined in previous papers. Color readings were made at 30 seconds with the Lovibond tintometer.

Fig. 1 shows the type of curve obtained with six different oils. Oils 1, 3, 4, 5, and 6 are good grade cod liver oils; Oil 2 is a ratfish liver oil. It can readily be seen that the intensity of blue color produced with the different oils is not a linear function of the concentration in any case; also that the type of curve and deviation from a linear function is different in each oil, so that no mathematical expression can be formulated which will express the curve

TABLE I.

Relative Color Values Obtained from Dilution Curves and from Tangents to the Curves of Several Fish Liver Oils.

Oil No.	25 per cent oil. Determination.		1 per cent oil. On tangent.	
	Reading.*	Ratio.	Reading.*	Ratio.
1	13.0	1.0	1.2	1.0
2	2.9	0.2	0.35	0.3
3	29.5	2.3	6.8	5.7
4	13.7	1.0	5.7	4.8
5	6.2	0.5	6.3	5.2
6	14.2	1.1	3.8	3.2

* Readings are given in Lovibond blue units.

for all of the oils. As pointed out in previous papers the dilution curves for the oils apparently approach a linear function at very low values of blue. If the color produced were a linear function of concentration it would coincide with the tangent to the curve at the origin. Tangents to the curves were drawn from the origin as nearly as could be estimated from the plotted values.

Table I gives a comparison of the blue color produced by 0.3 cc. of a 25 per cent solution (weight: volume per cent), corresponding to 0.075 gm. of oil per determination; and the value where the tangent to the curve at the origin crosses 1 per cent oil, corresponding to the value that would be produced by 0.003 gm. of oil if the color were a linear function. Relative values are also given for each set of figures calculated to consider Oil 1 as unity.

Drummond and Morton (3) have recently reported satisfactory comparison between the colorimetric and feeding experiments on six cod liver oils, where they apparently made color determinations at a single concentration of oil. As their tintometer

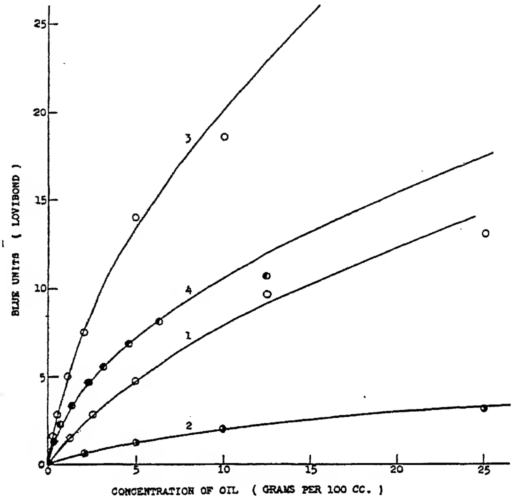

FIG. 2. Curves calculated by the formula $X = aY^2 + bY$ from the experimental data for four fish liver oils.

TABLE II.

Ratios of Slopes at Origin of Curves Calculated from the Formula, $X = aY^2 + bY$.

Oil No.	$\frac{dX}{dY}$ where $Y = 0$.	Ratio.
1	0.69	1.0
2	1.7	0.4
3	0.11	6.2
4	0.13	5.3

readings are not given we cannot tell at what concentration or color values the comparisons were made. They were probably fortunate in selecting six oils giving very similar dilution curves. It is obvious that a satisfactory comparison could not be made between the colors produced by the oils used in this paper at any single concentration, even though it be as low as 2 or 3 per cent.

Oils 1, 2, 3, and 4 give curves somewhat similar in type, and in attempting to correlate them, they were found, for values below 8 or 9 blue units, to approximate curves of the type formula $X = aY^2 + bY$, as shown in Fig. 2. The continuous curves represent the calculated values from the above formula. The circles give the experimental values obtained. From the formulæ of the curves the slope of the curve at the origin, which would represent a tangent at that point, may be calculated as $\frac{dX}{dY}$, where $Y = 0$. The values obtained for the slope of the curves at the origin are given in Table II together with the ratio of the corresponding blue produced per unit of oil compared to Oil 1. These values have a relationship similar to the tangent values estimated in Table I. These calculated values are probably more accurate as it is difficult to estimate an exact tangent to a curve. However, calculated values could not be obtained for Oils 5 and 6 as they did not follow any type formula tried.

By preliminary feeding experiments on these oils it is found that 0.006 gm. of Oil 1 per day causes an average gain in weight of 7.8 gm. per week over an 8 week period, with the method of Sherman and Munsell (4) for vitamin A. Under similar conditions 0.0011 gm. of Oil 3 gives a gain in weight of 7.6 gm. per week or a ratio in vitamin potency of the oils of approximately 1:5.5, a ratio similar to that for the tangents of the curves as opposed to 2.3 for the curve values at 25 per cent. With Oil 4, 0.0017 gm. gave an average gain in weight of 9.5 gm. per week, making it an oil only slightly less potent than Oil 3 as indicated by comparing the tangents and not equal to Oil 1 as would be predicted by the colors produced by a 25 per cent solution. Oil 5 was presented to us as a tested oil containing 1000 units of vitamin A per gm., which makes it exactly equivalent to Oil 3 as would be expected from the tangents to the curves, and not containing only half the amount of vitamin A present in Oil 1 as would be predicted from the color values at 25 per cent. The biological unit for Oil 3 is 0.99 mg.; that for Oil 2 is 29 mg.

Oil 5 is a highly flavored, refined oil giving an intense red with antimony trichloride reagent when the undiluted oil is used. The essential oil may be steam-distilled and the distillate gives a red with the reagent.

TABLE III.

Colors Produced by Chloroform Solutions of Essential Oils with Antimony Trichloride Reagent.

Name of oil.	Color of oil.	Colors produced by antimony trichloride.	
		Initial.	Final.
Oil of amber.	Yellow-green.	Brown.	Brown.
Aspic.	Colorless.	Yellow.	"
Bay leaf.	Yellow.	Yellow-green.	Yellow-green.
Bergamot.	Colorless.	Yellow.	Turbid.
Bitter almonds.	"	Colorless.	Colorless.
Cade.	Brown.	Brown.	Brown.
Cajeput.	Colorless.	Yellow-brown.	Turbid.
Camphor.	"	Colorless.	"
Caraway.	"	Yellow.	Salmon-pink.
Cassia.	"	Yellow-orange.	Yellow-orange.
Cedar wood.	"	Yellow.	Blue.
Cinnamon.	"	"	Brown.
Cloves.	"	"	Yellow.
Coriander.	"	"	Turbid.
Croton.	Yellow.	"	Orange.
Ethereal.	Colorless.	Pink.	Purple.
Eucalyptus.	"	Yellow.	Brown.
Juniper.	"	"	"
Lavendar.	"	"	Turbid.
Lemon.	"	"	Pink.
Menthol.	"	Colorless.	"
Nutmeg.	"	Yellow.	Salmon-pink.
Orange.	"	"	Yellow.
Peppermint.	"	"	"
Pimento.	Yellow.	"	"
Rosemary.	Colorless.	"	Pink.
Saffrol.	Yellow.	Yellow-green.	Yellow-green.
Sassafras.	"	"	"
Spearmint.	Colorless.	Yellow.	Red.
Sweet birch.	Red.	Pale yellow.	Pale yellow.
Thyme.	Colorless.	Pink.	Turbid.
Verbena.	"	Yellow.	Brown.
Wormseed.	"	"	Crimson.
Wormwood.	Greenish yellow.	Green.	Green.

As many of the cod liver oils on the market are flavored, and the essential oils contain unsaturated compounds which produce colors with antimony trichloride reagent, the colors produced by the chloroform solutions of several essentials oils with antimony trichloride were tested. The especial interest in essential oils was to determine if any would give a blue color which might be mistaken for the blue produced by fish liver oils. The results are given in Table III. Essential oils which were readily obtainable were used without regard to their value in flavoring fish oils. The essential oils give various shades of yellow, brown, and red, with one green and one purple, but only one of the oils tested, cedar wood oil, was found to give an intense permanent blue, which had an absorption band with a maximum at 580 $\mu\mu$.

Oil 6 is a crude cod liver oil intended for poultry feeding, developing a deep red color with high concentrations of the oil, and a marked deviation of the blue produced with antimony trichloride from a linear function. By feeding experiments it was found to be less potent than Oil 3, but much better than Oil 1.

Attempts were made completely to extract the unsaponifiable substance from a saponified cod liver oil. To weighed portions of about 5 gm. of oil were added 1.75 cc. of a potassium hydroxide solution (6.25 cc. of water to 6.5 gm. of potassium hydroxide) and 0.25 cc. of alcohol; the mixture was heated over boiling water for 8 minutes to saponify the oil, 0.5 cc. of water added, and the semi-solid soaps extracted with ethylene dichloride three times. The resulting solution, dried with anhydrous calcium chloride, was filtered and the ethylene dichloride evaporated at low temperature under reduced pressure in an atmosphere of either CO_2 or N_2. The residue was dissolved in petroleum ether and washed with water until it showed no further tendency to form an emulsion. The petroleum ether solution was then dried over anhydrous sodium sulfate and made up to a volume of 100 cc. Aliquots of the petroleum ether solution were evaporated at low temperature *in vacuo*, and the residue dissolved in chloroform in order to make the colorimetric readings. Chloroform solutions of the unsaponifiable substance from Oil 6 were made to represent various dilutions of the original oil. The blue color observed for these solutions, plotted against the gm. per 100 cc. of the original oil

represented by the extract, is shown in Curve E of Oil 6 in Fig. 1. Curve E is a composite of three separate extractions which showed very similar results; the five points determined fall on a straight line. Above 2.5 per cent the color produced is higher than with a similar concentration of the original oil. However it is not considered to be a quantitative extraction as it does not coincide with the tangent to the curve at the origin. Many of our extracts have shown high vitamin content by feeding experiments and given colors darker in blue units than an equal concentration of the original oil; however, we have never obtained complete extraction. Some of the blue-producing substance is always lost or destroyed during saponification, extraction, and washing.

A further study of the antimony trichloride test and comparison of the colorimetric and biological assay of vitamin A is being carried on in this laboratory.

SUMMARY.

1. A few of the various types of dilution curves which may be obtained with antimony trichloride and fish liver oils have been shown.

2. There is apparently no uniformity in type of dilution curve with various oils, and consequently it is impossible to make any comparison as to the vitamin A potency of the oil where the color produced is not a linear function of the amount of oil used.

3. If the color produced with any oil were a linear function of the vitamin present and therefore of the amount of oil used it would coincide with the tangent to the dilution curve at the origin.

4. The relative potency of vitamin A as shown by feeding experiments checks very closely with the relative color values of the tangent to the curve at the origin.

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FACTORS INFLUENCING THE DISTRIBUTION AND CHARACTER OF ADIPOSE TISSUE IN THE RAT.

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INTRODUCTION.

In animals, fats are widely distributed in the organs and tissues, but certain regions apparently serve as storehouses, for in these, large accumulations of fat are normally found. The amount of fat which is deposited in folds of connective tissue around the viscera, under the skin, and between the muscle sheaths varies largely in animals of apparently identical nutritive condition (Mayer and Schaeffer (1913, 1914–15) and Terroine and colleagues (1913, 1920, and 1922)). If these deposits are greatly reduced as a result of inanition, ptosis of many organs may result (Determan (1919) and Rubner (1920)).

At present there is little information regarding the normal distribution of the reserve fat or of its variation with physiological conditions. The determination of the effects of age, sex, chronic undernutrition, fasting, and exercise upon the depot fats should enable one to understand more fully the way in which fat is stored or mobilized in times of stress. Furthermore, it is conceivable that a variation in the composition of the stored fat might cause a difference in its distribution and utilization.

That the composition of the body fat depends upon the type of diet ingested has been shown by numerous investigators, including Hofmann (1872), Lebedeff (1882), Munk (1884), Winternitz (1898), Lummert (1898), Rosenfeld (1902), Lyman (1917), Ellis and Isbell (1926), Anderson and Mendel (1928), Eckstein (1929), and McAmis, Anderson, and Mendel (1929). Abderhalden and Brahm (1910) were of the opinion that the fat found in the protoplasm of the cell is not so influenced. More recently Terroine and Belin (1927) found that the type of fat fed previous to a fasting period had no effect upon the iodine number of the fatty acids of the *élément constant*. According to Shioji (1924–25), however, the fatty acids of the phospholipids as well as the total fatty acids of the kidney, liver, and heart of rabbits showed a slight change in iodine number when large quantities of a characteristic fat had been added to the diet. Sinclair (1929) likewise demon-

strated a change in the iodine number of the fatty acids of the phospholipids of the intestinal mucosa of animals that had been fed a characteristic fat. These conflicting results may be explained in part by the conditions of the experiments. Abderhalden and Brahm based their conclusions upon the determination of the melting point of the fats, a method too crude to detect small variations in composition, and the long fast to which Terroine's animals were subjected after the feeding period may have been responsible for his divergent conclusions.

The homogeneity of the adipose tissue of the body has been studied by a number of investigators. Rosenfeld (1902) and Lummert (1898) have reported the iodine numbers of the subcutaneous, omental, and intestinal fat of the dog. Schirmer (1921) reported the iodine numbers of the subcutaneous, mesenteric, and perirenal fat of rabbits and ducks fed olive oil or mutton tallow with the daily ration, whereas Henriques and Hansen (1901–02) have studied the chemical nature of the fat stored under the skin and within the abdominal cavity of animals of diverse species. In the dog, rabbit, and duck there was little difference in the iodine number of the subcutaneous and abdominal fat; in the horse, sheep, camel, and goose there was a greater difference; and in the hog and ox, a very great difference. In every case the subcutaneous fat was the most unsaturated. This fact was interpreted to mean that a difference in the temperature of the region in which the fat was deposited determined the degree of saturation of the fat—the more saturated fat being found in the warmer parts.

In the present communication data are presented to show the normal distribution and composition of the adipose tissue of rats ingesting different diets under varied physiological conditions. The distribution was determined by weighing the fat obtained from the various depots and the proportionate distribution was calculated from the results in terms of per cent of the total depot fat. An indication of the character of the fat was obtained through determination of the iodine value.

PART I.

THE INFLUENCE OF DIET, WEIGHT, AND SEX UPON THE DISTRIBUTION OF FAT.*

BY FUMIKO YAMAGUCHI, WILLIAM E. ANDERSON, AND LAFAYETTE B. MENDEL.

In order to study the effect of diet upon the distribution of the fat in the animal body, groups of rats were fed food mixtures

* The experimental data presented in this paper were taken from the thesis submitted by Fumiko Yamaguchi to the Faculty of Yale University, School of Medicine, in candidacy for the Degree of Doctor of Medicine, June, 1929.

equicalorically rich in fat or in carbohydrate. A commercial hydrogenated oil, Crisco, soy bean oil, mutton tallow, or corn-starch supplied 83 per cent of the total calories in the respective diets, which closely approximate the high fat and high carbohydrate diets of Smith and Carey (1923-24). The above dietary fats were selected for comparison because they varied greatly in

TABLE I.

Composition of Diets.

Type of diet.	Components.	Amount per kilo of diet.	Calories* per kilo of diet.	Total calories.	Amount of diet furnishing 1300 calories.
		gm.		*per cent*	*gm.*
Rich in carbohydrate.	Casein.	166	681	17.0	
	Corn-starch.	803	3292	83.0	
	Salts.†	31			
	Total..............	1000	3973	100.0	327
Rich in fat.	Casein.	300	1230	17.0	
	Fat (Crisco, mutton tallow, or soy bean oil).	643	5980	83.0	
	Salts.†	57			
	Total..............	1000	7210	100.0	180

400 mg. of brewers' yeast and 130 mg. (4 drops) of cod liver oil were fed daily apart from the food to each rat.

* The conventional calorie values: protein, 4.1; carbohydrate, 4.1; and fat, 9.3 were employed. Inasmuch as the casein and starch contained small adjuncts of fat, moisture, etc., the energy value calculations are approximate.

† The inorganic salt mixture was described by Osborne and Mendel (Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, **37**, 557 (1919)).

their fatty acid make-up. Each diet furnished a total energy intake of 1300 calories.

To study the influence of weight (age), sex, and diet fed *ad libitum*, upon the distribution of fat laid down in the depots, groups of rats were raised on the diets rich in carbohydrate (corn-starch) or on the hydrogenated vegetable oil (Crisco) to 50, 150, and 250 gm. of body weight, respectively.

EXPERIMENTAL.

Albino rats of both sexes, 21 days old and weighing approximately 40 gm., were kept individually in cylindrical wire mesh cages, and supplied with tap water *ad libitum*. At the outset of

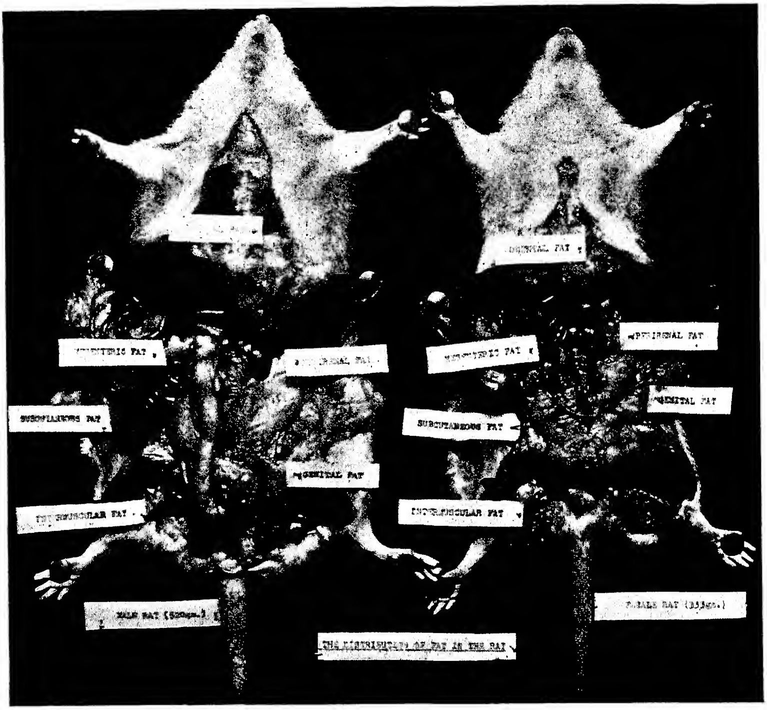

FIG. 1. Dissection of male and female rats to show the location of the fat deposits studied.

the experimental feeding period, they were subjected to 24 hours of fasting partially to deplete their depots of stored fat.

Table I indicates the composition of the experimental diets. These diets were comparable in that the ratio of the protein calories and that of the salt content to the total calories always remained constant regardless of the caloric value of the food.

Furthermore, the carbohydrate and the fat of the respective diets were present in equicaloric amounts. In other words, the fat and the carbohydrate were the only dietary variables. The protein was given in the form of casein fed at an effective, though not an optimal level (Osborne and Mendel, 1926). The indispensable vitamins were supplied apart from the daily ration in daily supplements of dried yeast powder and cod liver oil.

In preliminary trials, diets furnishing a total of 1300 calories were found sufficient to raise rats to body weights ranging from 135 to 175 gm.—sizes arbitrarily decided upon. All animals were weighed twice weekly. Food was withdrawn from each rat at least 4 hours previous to the act of killing with illuminating gas.

The fats indicated in Fig. 1[1] were removed from the six depots as follows:

Intermuscular.[2]—The fat was obtained from: (*a*) the region of the fore limb, posteriorly, within the crevice of the triceps lateralis and the spino-deltoideus muscles; (*b*) the lumbo-dorsal region, bilaterally, just below the inferior angle of the scapula, beneath the spino-trapezius and latissimus dorsi muscles; (*c*) the thoracic region, covering the intercostalis internus and transversalis abdominis muscles; (*d*) the inguinal region, directly under the superior portion of the gluteus superficialis muscle; (*e*) the posterior aspect of the thigh, below the biceps femoris anticus and posticus and the semitendinosus muscles.

Genital.—In the male, a broad band of adipose tissue attached to the testes and extending up into the abdominal cavity to the regions of the kidneys; in the female, attached along the uteri in the abdominal cavity and extending into the perirenal fat at the region of the kidneys.

Subcutaneous.—The panniculus adiposus and the pelt.

Perirenal.—The large mass of fat in which the kidneys are imbedded, extending from the diaphragm to the pelvis.

Mesenteric.—The broad fan-shaped fold of peritoneum containing the fat deposits and connecting the coils of the intestines with the posterior wall of the abdomen.

[1] Fig. 1 was kindly furnished by Miss L. L. Reed, senior author of Part II of this paper.

[2] The terminology adopted was that used in the "Anatomy of the wood rat" by Howell, Figs. 6 and 7, pp. 29 and 32, respectively.

TABLE II.

Distribution of Fat (Fatty Acids) in Male Rats Restricted in Energy Intake to 1300 Calories.

Mean values; extremes given in parentheses.

Diet.*	No. of rats.	Age.	Body weight.	Total fatty acids.	Proportion of depot fat (fatty acids) to total fat (fatty acids).				
					Intermuscular.	Genital and perirenal.	Subcutaneous.	Mesenteric.	Omental.
		days	*gm.*	*gm.*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Corn-starch.	11	52	146	7.17	10.5	16.9	63.3	6.4	2.7
		(51–54)	(133–157)	(6.76–7.64)	(8.0–12.2)	(13.0–20.2)	(58.2–71.0)	(4.4–7.4)	(1.2–6.4)
Crisco.	10	53	157	14.45	8.6	18.4	64.4	5.8	2.6
		(52–56)	(150–182)	(11.63–16.91)	(6.5–9.9)	(14.2–23.5)	(59.0–70.1)	(4.1–7.4)	(1.9–3.5)
Mutton tallow.	3	56	163	12.58	9.0	21.5	56.9	9.3	3.1
		(53–60)	(150–175)	(8.92–16.56)	(7.5–10.8)	(19.8–23.3)	(56.6–59.7)	(8.6–10.0)	(2.9–3.4)
Soy bean oil.	3	65	149	12.10	9.1	23.9	55.7	7.9	3.3
		(64–67)	(148–150)	(9.81–13.88)	(8.4–10.3)	(21.2–28.1)	(52.0–58.0)	(6.6–9.4)	(2.7–3.6)

* The diet was named according to the substance furnishing 83 per cent of the total calories.

Omental.—The fat around the lesser and the greater curvature of the stomach hanging down in front of the intestines and partly attached to the pancreas and to the spleen.

The fat of each depot was preserved in 95 per cent ethyl alcohol until subjected to saponification with an alcoholic potash solution. From the soaps formed, the fatty acids were liberated by the addition of concentrated hydrochloric acid, and extracted with ether. The ethereal solution of fatty acids was washed free of acid, dried with anhydrous sodium sulfate, and filtered. Finally, the ether was removed by distillation and the fatty acids dried to constant weight.

Result.

Approximately twice as much total fat (fatty acids) was deposited by rats fed a diet rich in fat (Crisco) as by animals that ate an equicaloric amount of a diet rich in corn-starch (Table II). The same tendency to relatively increased fat storage as a result of diets rich in fat rather than carbohydrate is shown in the comparative experiments on the mutton tallow and soy bean oil rations. The deposition of greater amounts of fat by rats fed rations rich in fat is in accord with findings reported in Part II and serves to answer the question raised earlier by Rosenfeld (1902) concerning the relative fattening values of fat and carbohydrate. Whether the results obtained with corn-starch would apply in equal degree with other carbohydrates, notably levulose, remains to be determined.

A comparison of the proportionate distribution of the fat shows that the differences between the groups on the various diets are less than the variations of the individuals of any one group. Evidently, the distribution of the depot fat is independent of the diet.

Again in the experiments upon rats (either male or female) growing to body weights of 50, 150, and 250 gm. respectively, fed *ad libitum* on the Crisco or corn-starch diet, more fat was deposited by the animals raised on the diet rich in fat than on a regimen rich in carbohydrate. This is evident from the data included in Table III. On the Crisco diet, there was a relative increase in the proportion of fat deposited as the rats gained in

TABLE III.

The Influence of Diet, Weight, and Sex upon the Amount and Distribution of Fat in the Animal Body.

Rat No.	Body weight.	Age.	Sex.	Total fatty acids.	Total fatty acids. Proportion of body weight.	Proportion of depot fat (fatty acids) to total depot fat (fatty acids). Intermuscular.	Genital.	Subcutaneous.	Perirenal.	Mesenteric.	Omental.
Corn-starch diet.											
	gm.	*days*		*gm.*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
72, 74, 77	50	25	♂	2.55	5.09	3.5	3.9	85.0	3.7	2.6	1.2
78, 79, 83	50	25	♀	2.81	5.61	3.3	3.0	88.8	2.3	1.8	0.7
101	150	48	♂	7.12	4.75	8.8	9.8	63.8	7.7	6.7	2.9
93	150	48	♂	8.23	5.46	9.1	10.6	64.6	8.0	5.3	2.2
94	150	52	♀	7.97	5.31	8.0	12.0	65.8	6.6	5.6	1.9
102	150	50	♀	7.42	4.97	9.0	11.3	64.4	6.0	6.2	2.9
51	250	92	♂	28.75	11.48	8.9	11.9	55.6	14.1	7.5	2.0
52	250	87	♂	28.14	10.67	6.2	12.2	58.1	14.0	7.0	2.4
53	250	142	♀	29.15	11.66	2.8	19.0	63.1	8.4	5.1	1.6
54	250	148	♀	24.34	10.14	3.0	17.4	64.1	7.4	5.2	2.8
Crisco diet.											
71, 73, 75	50	25	♂	2.61	5.22	3.1	5.5	84.1	3.3	3.0	1 0
76, 81, 82	50	25	♀	3.04	6.09	2.8	3.6	87.0	3.2	2.4	0.8
92	150	48	♂	14.62	9.70	5.6	10.8	67.0	7.9	5.6	3.1
103	150	50	♂	13.64	9.10	6.6	11.4	63.7	9.0	6.5	2.7
91	150	49	♀	13.71	9.14	6.8	13.6	63.3	8.4	5.6	2.2
104	150	43	♀	13.54	9.03	7.0	12.2	64.3	8.0	5.8	2.5
55	250	98	♂	35.10	14.02	5.3	13.9	54.3	18.3	5.1	3.1
57	250	95	♂	33.70	13.50	6.0	13.3	53.5	19.4	4.8	2.9
56	250	145	♀	30.40	12.14	6.9	15.7	55.3	13.6	5.4	3.0
58	250	137	♀	31.70	12.92	4.6	19.0	61.7	9.0	4.0	2.2

weight, whereas, on the corn-starch diet, the proportionate amounts of fat deposited by rats of either 50 or 150 gm. body weight were about equal.

The data showing the proportion of depot fat to total fat are summarized in Table III. The proportion of the total fat in the various depots is not modified to any appreciable extent by the diets used. The proportion of depot fats to total fat was influenced more by the factors of size and sex than by diet. As the rats increased in body weight, the proportion of fat stored in the subcutaneous tissues tended to decrease.

The proportion of perirenal fat increased in both sexes with increasing body weight—about twice as much fat was stored in the perirenal depot by male rats of 250 gm. body weight. The accumulation of genital fat by the heaviest females was greater than by males of the same weight, regardless of the dietary régime. The largest proportion of intermuscular fat was deposited by rats of both sexes, raised to 150 gm. body weight on the corn-starch diet.

SUMMARY.

Influence of Diet.

In every case more fat was stored by rats ingesting rations rich in fat than by those on a diet rich in the carbohydrate, corn-starch. The proportionate distribution of the stored fat was similar for rats of the same sex and weight, regardless of the type of diet fed.

Influence of Weight.

As the rats of both sexes upon both diets increased in body weight, they stored more fat in proportion to body weight (with the single exception of rats of 150 gm. body weight ingesting the corn-starch diet which showed no increase over the rats of 50 gm. body weight). The proportion of subcutaneous fat was largest in small rats (50 gm. body weight).

Influence of Sex.

Male rats of 250 gm. body weight stored more fat in the perirenal fat depot than female rats; the latter tended to store more fat in the genital fat depot.

PART II.*

THE INFLUENCE OF DIET, UNDERNUTRITION, FASTING, AND ACTIVITY UPON THE DISTRIBUTION AND CHARACTER OF FAT.

By LUCILLE L. REED,† WILLIAM E. ANDERSON, AND LAFAYETTE B. MENDEL.

The present communication was designed to answer a number of questions:

What is the distribution of stored fat in animals at the end of the period of most rapid gains in weight?

Is the distribution or absolute amount of fat affected by the type of diet ingested?

Will an animal deprived of food, or limited in its food intake, or forced to exercise, show a preferential utilization of the fat from any particular store? Does augmented muscular activity influence the distribution of stored fat, regardless of its composition?

What is the chemical nature of fat that is stored: does the location of the fat in the body determine its degree of saturation?

Is the degree of saturation of the fat of adipose tissue altered by muscular activity, or by deprivation or limitation of food—conditions that may cause draughts upon the body stores of fat?

EXPERIMENTAL.

The diets used in this investigation were those designed by Anderson and Mendel (1928) to supply in constant proportions the protein, inorganic salts, and vitamins indispensable to adequate nutrition in rats, while varying the possible precursors of fat formation. The diets were designated according to that substance furnishing 60 per cent of the total calories; for example, the starch diet, the soy bean oil diet, and the coconut oil diet. Details of the composition of these diets are given in Table IV.

Female albino rats of approximately 35 to 45 gm. body weight

* Part of the expenses of this investigation was defrayed by a grant from the Russell H. Chittenden Research Fund for Physiological Chemistry.

† Sterling Fellow in Yale University (1929–1930).

at 21 days of age were kept individually in false bottom cages, weighed twice weekly, and fed one of the above diets. Furthermore, the rats on each diet were divided into groups which were subjected to various experimental conditions, as follows:

TABLE IV.

Composition of Experimental Diets.

Basal Diets.

Diet.	Components.	Amount.	Energy value.	Total calories.
		gm.	*calories*	*per cent*
Starch.	Dried skimmed milk.	42.7	156	40
	Corn-starch.	57.3	235	60
	Total..................	100.0	391	100
Soy bean oil.	Dried skimmed milk.	62.8	230	40
	Soy bean oil.	37.2	346	60
	Total..................	100.0	576	100
Coconut oil.	Dried skimmed milk.	62.8	230	40
	Coconut oil.	37.2	346	60
	Total..................	100.0	576	100

*Accessories.**

To rats under 100 gm. of body weight on each of the above diets:

Brewers' yeast.............................. 400 mg.

Cod liver oil................................ 35 " (1 drop).

To rats over 100 gm. of body weight on each of the above diets:

Brewers' yeast.............................. 800 mg.

Cod liver oil................................ 105 " (3 drops).

* The yeast and cod liver oil were mixed together in a small dish and fed once daily. They were consumed immediately in every case.

Under Conditions of Inactivity.

Calories Unlimited.—These rats ate the diet *ad libitum* until they attained 180 to 190 gm. of body weight and were killed.

Calories Limited.—These rats were limited daily to 70 per cent of the food intake of the animals in the above group and killed at the same age as the above group.

Fasting.—Food was eaten *ad libitum* until the rats attained 180 gm. of body weight; subsequently, they were given only the

vitamins and water until the body weight had decreased 30 per cent.

Under Conditions of Activity.

Voluntary Activity during the Day.

Calories Unlimited.—The rats were placed for 7 to 8 hours daily in revolving cages which were equipped with a counter to record the number of revolutions made. The diet was eaten *ad libitum* until the rats were killed at 180 to 190 gm. body weight.

Calories Limited.—The rats were placed for 7 to 8 hours daily in revolving cages. Their total food intake was restricted to that amount consumed by the individuals of the inactive group

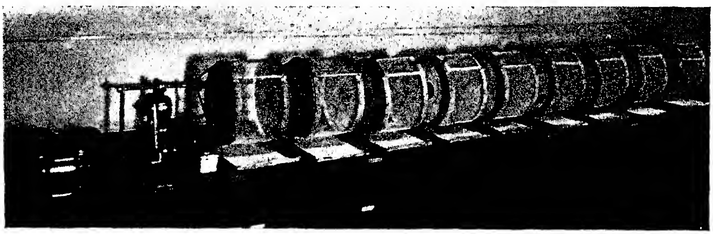

Fig. 2. The apparatus in which rats were forced to exercise.

listed as "Calories unlimited." The rats were killed when they attained the same age as the latter group.

Voluntary Activity during the Night.

Calories Unlimited.—The animals were placed daily in revolving cages between the hours of 10 p.m. and 8 a.m. They were fed *ad libitum* the soy bean oil diet until they reached 180 gm. of body weight.

Forced Activity during the Day.

Calories Unlimited.—Inasmuch as the voluntary activity of the individual rats in the above groups varied considerably in amount, all rats were subjected to a uniform amount of exercise. This was accomplished by attaching the individual cages to a motor-driven shaft permitting rotation at a speed of 11 revolutions per minute.

Furthermore, with the aid of a timer, the cages were intermittently rotated and held stationary for 2 minutes and $1\frac{1}{4}$ minutes respectively (Fig. 2). 2100 to 2200 revolutions per day were required of

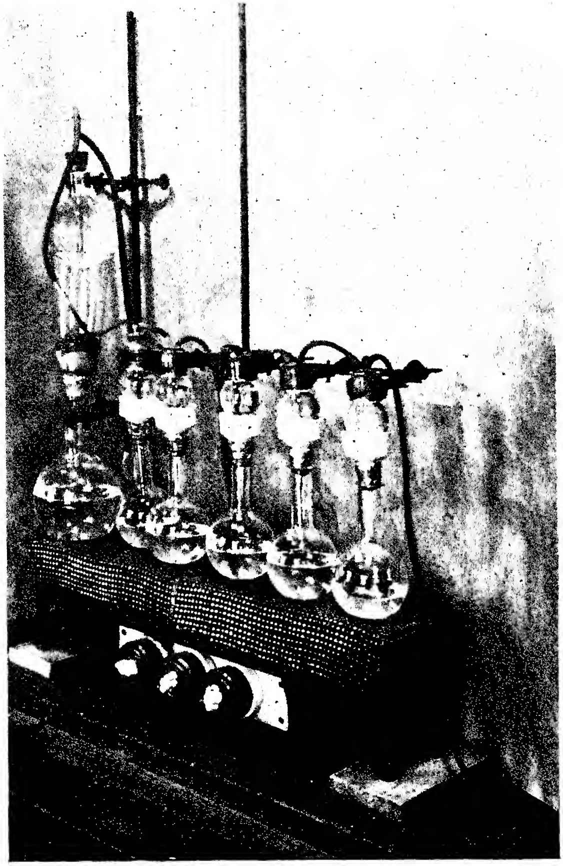

FIG. 3. Apparatus for the extraction of fat from the adipose tissue. The larger unit at the left is the modified Clark extractor; the smaller units show the authors' modifications.

the rats. Although the food intake was unlimited, these rats gained in weight very slowly, failing in many cases to reach 180 gm. of body weight.

All rats were killed by illuminating gas and carefully dissected as described in Part I with the following exceptions: In no case was the fat taken from between the intercostalis internus and transversalis abdominis muscles; the subcutaneous fat was clipped free of the pelt which was discarded. The depots selected are pictured in Fig. 1. The dissected tissue was preserved in 95 per cent alcohol until extracted with hot alcohol and ether.

The apparatus for the extraction of fat consisted of a modified Clark extractor, used by Bloor (1926) for the separation of lipids from animal tissues. The subcutaneous fat was cut up finely with scissors and extracted according to the Bloor procedure. The smaller depots after being well cut up were wrapped in hardened filter paper and placed in 6 cm. Buchner funnels which were substituted for the percolator part of the Bloor apparatus (Fig. 3). Any material adhering to the scissors was washed onto the depot fat with a stream of redistilled ether, the Florence flask half filled with 95 per cent alcohol, and the extraction begun. Extraction was considered complete when at the end of 5 to 24 hours the residue assumed a dry paper-like consistency. Saponification of the pooled residue from the depots of four rats yielded the results indicated below. The concentration and proportion of reagents were those used by Hertwig, Jamieson, Baughman, and Bailey (1926). After saponification and acidification, the solution was extracted with ether and the ether-soluble material dried to constant weight.

Analyses Indicating the Value of the Extraction Method Used.

Fat depot.	Total fat removed by extraction.	Residue.	Ether-soluble matter removed after saponification of residue.	Proportion of fat unextracted from the depot.
	gm.	*gm.*	*gm.*	*per cent*
Intermuscular	4.266	1.1	0.005	0.11
Genital	16.852	0.7	0.003	0.02
Subcutaneous	52.153	9.0	0.006	0.01
Perirenal	16.557	1.0	0.005	0.03
Mesenteric and omental	11.804	1.2	0.001	0.01

The extremely small amount of ether-soluble material obtained from the saponification of the residue indicated the efficiency of the method, and justified the acceptance of the paper-like appearance of the residue as indication of complete extraction.

The alcohol-ether solution of the fat which had been extracted from the adipose tissue was placed in 500 cc. balloon flasks, immersed in a water bath between 40° and 50°, and the solvent distilled off under reduced pressure. The residue was taken up in redistilled ether, the ethereal solution dried over anhydrous sodium sulfate, centrifuged free of the dehydrating agent, and poured into a weighed Erlenmeyer flask. The ether was driven off on a water bath and the fat dried in a stream of carbon dioxide at 80°. The Erlenmeyer flask was weighed after the carbon dioxide had been removed by evacuation and the weight of the fat obtained by difference. The iodine number was determined in duplicate by the Hanus method.

No precaution was taken to keep the fat in an atmosphere of carbon dioxide while it was in alcohol or ether solution, inasmuch as Bloor (1926) had found the vapors of the solvent used in extraction are as adequate protection from oxidation as is an atmosphere of carbon dioxide. To test the validity of this claim, two samples of the soy bean oil, weighing approximately 5 and 1 gm. respectively, were spread upon filter paper, extracted for 4 hours with alcohol in the usual manner, allowed to stand overnight in ether, and again extracted with alcohol for 5 hours. The combined alcoholic solutions of fat were concentrated and the fat taken up in ether as usual. 4 days elapsed before the fat was dry and ready for the iodine number determination. The iodine number of the original oil was determined at the same time as that of the two samples. The data obtained are recorded below.

	Iodine No.			
	Determination No.			Average.
	1	2	3	
Original oil	130.3	129.9		130.2
5 gm. sample	129.3	128.4	129.2	128.9
1 " "	130.5	131.4		130.9

These data are interpreted to indicate that oxidation plays no appreciable rôle in the extraction of fat by this method.

DISCUSSION.

A study of the v luntary activity of all the rats reveals first, the enormous amount of individual variation of the rats in each group, and secondly, the striking amount of activity exhibited by those rats on the soy bean oil diet that were allowed to run at

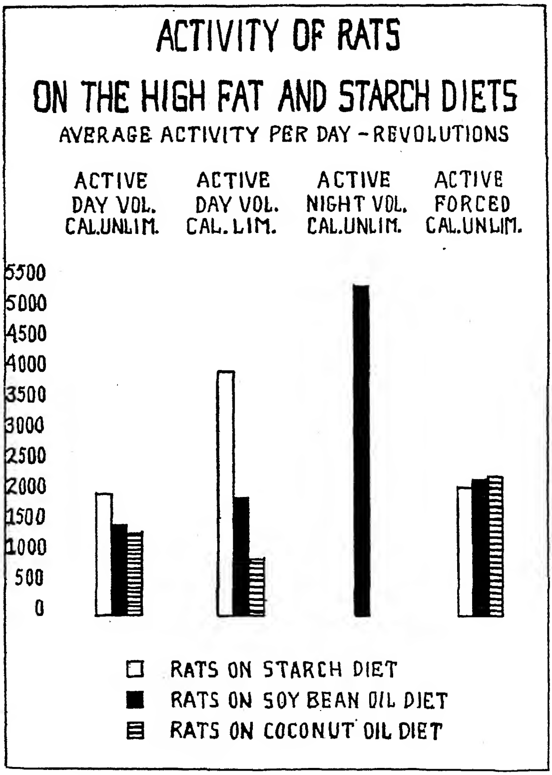

CHART 1.

night (Chart 1). Although the latter group ate more food than any other group of rats on the soy bean oil diet and increased in body weight to 180 gm., they had even less fat than those rats on the soy bean oil diet which had been fasted until they lost 30 per cent of their original body weight. At periods of great activity several of these rats must have run constantly at a rate of 20

revolutions per minute for the 10 hour period they were in the cages. Slonaker (1925) had observed even greater nocturnal activity on the part of female rats of 5 months of age and over. The most active of the rats running by day were on the starch diet, less active were those on the soy bean oil diet, and least, those on the coconut oil diet.

The aim to make each rat indulge in the same amount of exercise was not realized in the strictest sense, inasmuch as the rats subjected to forced running held on to the wire side of the cage part of the time. All the rats clung to the cage as the latter began to rotate, and some continued to cling for a good portion of the time, while others ran in the bottom of the cage. The amount of work involved in hanging on upside down, or in running in the bottom of the cage may not have been widely different, and the distribution of kinetic and static exercise among the rats of the different dietary groups was probably about the same.

When the rats of the inactive group were allowed to eat *ad libitum*, they ingested daily equicaloric amounts of any diet. The starch diet was less efficient in bringing the rats to 180 gm. of body weight than either of the fat diets. As a consequence, the rats on the starch ration ate the diet over a longer period, and ingested more total calories. In spite of the fact that these rats obtained more calories, they stored less fat in proportion to body weight than the rats ingesting the high fat diets (Chart 2). This is in accord with the findings in Part I.

As would be expected, that group of rats limited to 70 per cent of their normal daily food intake had a much lower body weight than the rats eating *ad libitum*, but the proportion of stored fat to body weight was equal to that of the rats not stinted in their food intake. Perhaps the level at which the diet was fed provided only enough protein for the amount of growth exhibited, while the large proportion of calories, furnished by the starch, or fat of the diets, being in excess of the energy requirements of the body, was converted to fat and stored.

The rats which were forced to exercise were thin in spite of an unlimited food supply (Fig. 4). Possibly they were unable to eat and digest enough food to maintain body weight under the conditions imposed. This was undoubtedly true of the particular group

of rats on the soy bean oil diet running at night, which ate more food than any other group of rats on this diet (Chart 2).

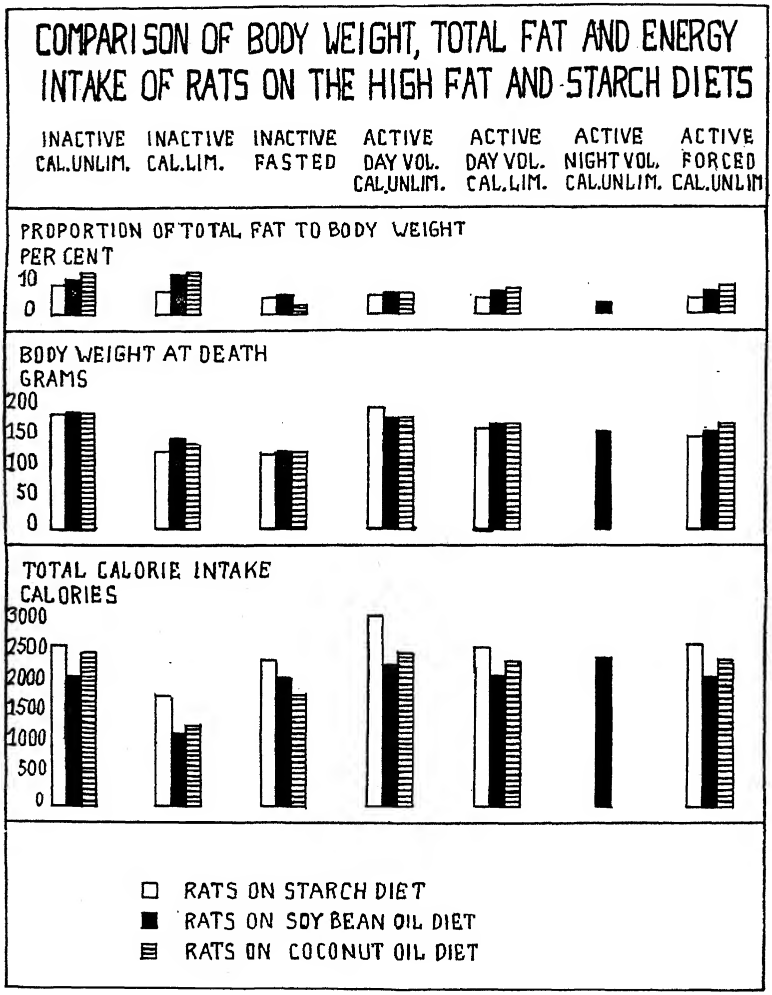

Chart 2.

The distribution of stored fat was the same for rats on any diet when the rats were allowed to eat ad libitum, and were not not forced

to run (Tables V to VII). This also is in accord with the findings in Part I. Forced exercise and nocturnal running appeared to leave a higher proportion of fat between the muscle sheaths of rats on any diet. It is of interest that the genital fat of those rats fed the starch diet which were limited daily to 70 per cent of their normal food intake, was, like that of the rats forced to exercise, reduced in proportion with a corresponding rise in the proportion of subcutaneous fat. This difference in fat storage between the rats on the starch diet and those of the other two diets was demonstrated in the "Forced exercise" and "Inactive calories limited" groups only.

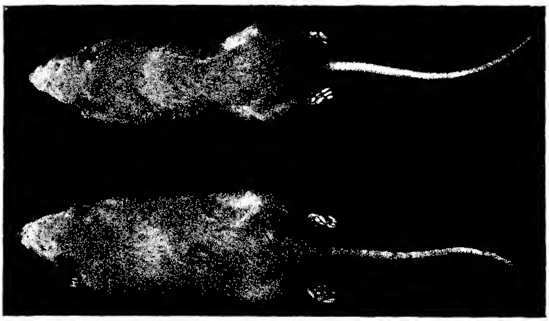

Fig. 4. The effects of exercise upon the appearance of the rats are shown in the animal at the top which ran approximately 93 miles in 70 days and ingested the same amount of food as the litter mate control shown below.

Rats raised on the starch, soy bean oil, and coconut oil diets stored fat with iodine numbers respectively, of 50 to 60, 100 to 124, and 25 to 35. The iodine numbers of the dietary oils were 130.1 for the soy bean oil, and 8.2 for the coconut oil (Tables VIII to X).

When the rats on the coconut oil diet were fasted until they lost 30 per cent of their body weight, they were depleted of a greater proportion of fat than any rats fasted to the same extent. The fat which was present had a slightly higher iodine number than the stored fat of any of the rats on this diet. This rise in iodine number may be a result of the relative increase of phospholipid material in the extracted fat. Assuming that the concentration

TABLE V.

Distribution of Fat in the Animal Body.

Starch Diet.

The values are given in per cent.

Group.	Rat No.	Proportion of depot fat to total depot fat.						Proportion of total fat to body weight.
		Intermuscular.	Genital.	Subcutaneous.	Perirenal.	Merenteric and omental.		
Inactive, calories unlimited.	1	5.1	21.8	47.7	15.0	10.4		8.7
	6	6.8	16.1	50.6	11.7	11.8	4.0	9.4
	8	4.1	25.4	41.5	13.6	15.4		7.8
	11	5.1	20.7	47.0	11.1	16.1		8.6
	15	5.4	17.0	49.9	14.8	9.6	3.3	13.6
Average..................		5.3	20.2	47.3	13.2	14.1		9.6
Inactive, calories limited.	40	5.3	12.9	59.8	10.3	8.2	2.5	7.1
	41	6.2	10.8	60.8	9.5	7.8	3.9	6.8
	42	4.4	13.2	53.6	16.2	8.2	4.4	9.4
Average..................		5.3	12.3	58.1	12.0	8.1	3.6	7.8
Inactive, fasting.	36	5.3	18.7	55.3	10.3	5.3	5.1	5.3
	38	5.3	22.3	53.5	9.4	5.9	3.6	5.5
	39	4.2	27.7	45.7	7.9	9.9	4.6	5.7
Average..................		4.9	22.9	51.4	9.2	7.0	4.4	5.5
Active, calories unlimited, day, voluntary.	2	6.0	29.7	40.5	12.8	12.0		7.2
	5	4.8	24.0	45.4	9.4	16.4		6.0
	9	5.4	17.0	51.7	9.2	16.7		4.2
	13	5.4	17.4	51.0	9.6	16.6		7.5
Average..................		5.4	22.0	47.2	10.2	15.4		6.2
Active, calories limited, day, voluntary.	4	5.5	13.9	60.9	5.3	13.4		1.4
	7	5.6	16.1	53.5	8.4	16.4		5.7
	10	6.0	19.3	51.6	8.4	14.7		6.3
	14	5.1	21.6	49.8	11.3	8.8	3.4	6.6
	16	5.8	18.4	50.6	9.5	15.7		7.1
Average..................		5.6	17.9	53.5	8.6	14.5		7.8
Active, calories unlimited, forced.	67	8.9	17.8	47.8	10.4	11.8	3.3	6.6
	68	8.7	9.6	40.8	27.5	9.3	4.1	3.4
	69	9.1	16.6	54.2	10.0	10.0		3.3
	87	8.7	12.6	63.6	6.1	7.1	1.4	7.7
	88	13.1	4.8	60.3	5.8	11.9	4.1	4.7
Average..................		9.7	12.5	53.3	12.0	10.0	3.2	5.2

TABLE VI.

Distribution of Fat in the Animal Body.

Soy Bean Oil Diet.

The values are given in per cent.

Group.	Rat No.	Proportion of depot fat to total depot fat. Intermuscular	Genital	Subcutaneous	Perirenal.	Mesenteric	and omental.	Proportion of total fat to body weight.
Inactive, calories unlimited.	24	5.5	15.9	58.1	9.5	7.5	3.5	15.5
	27	4.9	19.4	53.0	12.2	8.0	2 5	16.0
	28	6.0	21.4	37.6	20.2	10.6	3.2	7.1
	31	4 9	20.2	46.1	14.5	10.9	3.4	10.0
	35	4 9	20.7	42.3	15.9	11 2	4.9	12.1
	73	6.0	20.4	51.1	11.8	7.8	2.9	13.7
	74	6 2	22.8	48.5	13.6	6.2	2.7	9.4
	76	4.9	22 5	41.3	18 3	9.9	3.1	11.3
	77	5 1	19.3	50.7	13.9	8.5	2.5	9.8
	78	6.3	26.6	42.5	16.3	8.2		9.7
	83	5.3	23.3	37.1	13.5	18.0	2.7	13.3
Average.		5.5	21.1	46.2	14.3	9.7	3.1	11.6
Inactive, calories limited.	103	6.1	25.6	45.5	13.8	6.9	2.1	9 8
	104	5.9	18.6	54.9	10.6	7.4	2.6	11.7
	105	4.8	18.6	56.1	11.1	6.8	2.6	17.9
Average.		5.6	21.3	51.5	11.8	7.0	2.4	13.1
Inactive, fasting.	34	6.7	22.4	52.7	10.4	3.5	4.3	2 3
	26	5.2	19.1	55.0	12.8	3.7	4.2	1.9
	33	3.7	19.8	50.7	12.9	8.6	4.3	11.7
Average.		5.2	20.4	52 2	12.0	5.3	4.3	5.3
Active, calories unlimited, day, voluntary.	18	7.3	17.1	49.8	12.1	9.1	3.6	9.5
	20	7.8	32.8	40.0	10.6	6.5	2.3	14.4
	23	5.2	27.5	45.4	11.1	7.4	3.4	6.7
	32	4.8	19.3	45.5	13.3	12.5	4.5	4.7
Average...................		6.3	24.2	45.2	11.8	8.9	3 3	8.8
Active, calories limited, day, voluntary.	17	6.3	17.3	46.3	14.3	13.2	2.6	4.6
	19	5.0	16.4	51.0	12.7	11.8	3.1	9.9
	22	5.1	24 8	40.5	12.5	13.8	4.3	7.8
	25	5.2	25.6	46.7	13.2	7.8	2.8	10.9
	29	6.5	15.1	56.1	10.1	13.1		5.7
Average..................		5.6	19.8	48.1	12.6	11.6	3.2	7.8

TABLE VI—*Concluded.*

Group.	Rat No.	Proportion of depot fat to total depot fat. Intermuscular.	Genital.	Subcutaneous.	Perirenal.	Mesenteric and omental.		Proportion of total fat to body weight.
Active, calories unlimited, night, voluntary.	80	6.6	21.2	49.9	10.6	7.8	3.9	3.5
	81	10.2	19.6	36.0	18.2	8.5	7.5	1.9
	82	8.2	27.6	42.0	11.3	6.1	4.8	2.8
	95	7.9	16.3	51.8	12.2	7.8	4.0	3.5
	96	7.3	20.5	50.2	9.8	7.0	5.2	7.1
Average		8.0	21.0	46.0	12.4	7.4	5.1	3.7
Active, calories unlimited, forced.	70	10.0	22.9	40.2	13.1	11.2	2.6	7.6
	71	6.4	31.0	38.5	11.6	9.1	3.4	7.2
	72	11.8	19.5	45.7	10.7	7.5	4.8	9.8
	84	10.0	9.7	58.0	9.6	8.6	4.1	6.9
	85	7.8	12.7	59.3	10.2	6.9	3.1	4.9
	86	6.6	15.5	55.7	12.6	7.1	2.5	10.3
Average		8.8	18.5	49.6	11.3	8.4	3.4	7.8

of this substance with a higher iodine number than the stored fat does not change (Terroine and Belin, 1927) a depletion of the stored fat would tend to give a mixture with a higher iodine number.

Inasmuch as the iodine numbers of the different groups showed less variation than the members of any one group, it is concluded that none of the conditions imposed, except the fasting of the coconut oil group, caused a change in the chemical nature of the fat depots. Therefore, an average of all the values for one depot has been made in an effort to ascertain the rôle played by location in the chemical composition of the stored fat (Tables VIII to X). One is struck by the marked similarity in iodine values of the various depot fats of any rat—the difference between them being less than the variations between the iodine numbers of any depot obtained from different rats within the same group. The chemical nature of fat is apparently not coincident with its position in the body—which is in accord with the findings on the dog and duck, but in opposition to those on the hog (Henriques and Hansen,

TABLE VII.

Distribution of Fat in the Animal Body.

Coconut Oil Diet.

The values are given in per cent.

Group.	Rat No.	Proportion of depot fat to total depot fat.						Proportion of total fat to body weight.
		Intermuscular.	Genital and	subcutaneous.	Perirenal.	Mesenteric and	omental.	
Inactive, calories unlimited.	44	4.9	19.0	55.4	10.4	7.5	2.5	15.4
	45	3.1	15.9	60.0	10.1	7.4	3.5	15.7
	52	4.3	21.4	54.1	9.7	8.0	2.5	19.1
	56	4.8	22.7	49.7	12.5	8.0	2.3	14.4
	59	5.7	19.5	47.7	13.8	8.9	4.4	8.1
	97	4.6	20.1	52.0	13.1	7.0	3.2	11.6
Average.		4.6	19.8	53.2	11.6	7.8	3.1	14.1
Inactive, calories limited.	100	4.3	14.2	61.4	10.8	6.0	3.3	11.5
	101	4.2	26.0	50.7	10.6	6.6	1.9	17.5
	102	5.3	23.5	51.8	10.3	5.9	3.2	13.3
Average.		4.6	21.2	54.6	10.6	6.2	2.6	14.1
Inactive, fasting.	98	9.4	27.8	38.2	14.6	5.6	5.4	2.9
	99	9.4	27.2	36.7	12.2	6.8	7.7	2.9
Average.		9.4	27.5	37.5	13.4	6.2	6.5	2.9
Active, calories unlimited, day, voluntary.	49	6.9	23.9	45.3	11.9	12.0		4.8
	50	7.3	19.3	49.1	11.4	9.4	3.5	4.6
	54	6.8	24.6	41.4	18.0	4.4	4.8	9.7
	58	8.6	68.4		11.3	8.9	2.8	9.8
Average.		7.4	22.6	45.3	13.1	7.6	3.7	7.2
Active, calories limited, day, voluntary.	43	6.7	17.1	55.3	10.5	7.8	2.6	9.9
	48	6.5	15.7	54.6	10.9	7.6	4.7	7.4
	51	4.8	25.2	49.5	13.3	5.0	2.2	9.0
	53	5.7	26.2	46.3	8.5	10.1	3.2	9.1
Average.		5.9	21.1	51.4	10.8	7.6	3.2	8.9
Active, calories unlimited, forced.	64	6.1	25.8	44.0	16.0	5.6	2.5	11.1
	65	5.9	21.0	53.0	12.8	4.7	2.6	11.9
	66	9.1	19.8	50.1	10.8	7.8	2.4	5.1
	93	6.8	20.1	47.7	10.5	9.5	5.4	5.7
	94	5.2	23.1	47.8	12.4	8.6	2.9	12.9
Average.		6.6	22.0	48.5	12.5	7.5	3.2	9.5

TABLE VIII.

Iodine Values of Fat in the Animal Body.

Starch Diet.

Values indicate gm. of iodine absorbed per 100 gm. of fat (Hanus method).

Group.	Rat No.	Intermuscular.	Genital.	Subcutaneous.	Perirenal.	Mesenteric and	omental.
Inactive, calories unlimited.	1	57.2	59.2	57.8	52.8	50.3	
	3	60.0	56.5	57.2	54.7	50.3	
	11	58.5	52.0	54.3	53.8	47.0	50.0
	6, 8, 15	52.8	54.0	56.0	53.7	51.8	
Average		57.1	55.4	56.6	53.8	50.2	
Inactive, calories limited.	40, 41, 42	55.5	50.8	56.5	54.5	52.3	55.1
Inactive, fasting.	36	49.8	48.4	50.4	51.7	47.0	44.5
	38	58.7	52.9	56.6	53.0	50.9	55.7
	39	57.5	55.8	56.6	53.9	49.6	53.5
Average		55.3	52.4	54.5	51.9	49.2	51.2
Active, calories unlimited, day, voluntary.	2	62.1	60.2	59.5	54.8	52.2	
	13	59.5	58.1	55.7	53.3	52.3	49.6
	5, 9	51.1	55.4	57.6	55.9	51.0	
Average		57.6	57.9	57.6	53.7	51.4	
Active, calories limited, day, voluntary.	4		62.5	56.5			
	7	56.4	58.0	61.0	57.1	57.8	
	16	58.2	53.5	58.5	57.0	55.4	56.4
	10, 14	53.9	53.6	54.3	54.0	49.0	
Average		56.2	56.5	56.8	56.0	54.2	
Active, calories unlimited, forced.	67	53.6	55.4	56.6	54.8	53.3	52.7
	68	52.4	52.4	54.5	48.6	51.9	47.3
	69	54.2	56.0	58.1	54.1	54.9	
	87	51.7	53.0	53.2	52.0	50.0	46.4
	88		46.0	56.5	52.4	49.7	42.3
Average		53.0	52.6	55.8	52.4	52.0	47.4
Average values for all groups		55.8	54.3	56.3	53.7	51.4	51.6

TABLE IX.

Iodine Values of Fat in the Animal Body.

Soil Bean Oil Diet.

Values indicate gm. of iodine absorbed per 100 gm. of fat (Hanus method).

Group.	Rat No.	Inter-musculo-lar.	Genital.	Subcu-taneous.	Peri-renal.	Mesen-teric.	Omental.
Inactive, calories unlimited.	73	109.1	118.5	103.5	117.1	118.5	113.0
	74		124.1	115.8	119.5		
	76						93.1
	77	101.2	108.7	107.0			95.3
	78			116.5		100.3	
	83		104.8	114.8		111.2	102.0
Average.................		105.1	114.0	111.3	118.3	110.0	100.9
Inactive, calories limited.	103	115.2	113.5	115.3	113.0	105.7	111.3
	104	105.4	115.8	116.2	119.2	119.5	
	105	116.1		113.1	117.1	112.0	103.5
Average.................		112.2	114.7	114.8	116.4	112.4	107.4
Active, calories unlimited, night, voluntary.	80						
	81	105.2	109.7	109.8	108.9	113.8	110.4
	82	113.4	118.1	116.6	111.4	98.7	104.0
	95	107.0	103.1	113.8	104.5	108 2	92.2
	96	97.0	111.4	114.4	106.6	111.9	105.1
Average.................		105.6	110.6	113.6	107.9	108.2	102.9
Active, calories unlimited, forced.	70						
	71	120.6	123.6	118.4	122.1	122.0	115.1
	72	113.5	112.8	113.9	110.4	110.3	105.0
	85						
	86	109.4	115.2	118.3	113.8	115.3	112.0
Average.................		114.3	114.4	116.7	113.0	111.3	109.8
Average values of all groups................		109.3	113.4	114.1	113.9	110.5	105.2

The iodine number of the food fat, soy bean oil, is 130.1.

TABLE X.

Iodine Values of Fat in the Animal Body.

Coconut Oil Diet.

Values indicate gm. of iodine absorbed per 100 gm. of fat (Hanus method).

Group.	Rat No.	Inter-muscular.	Genital.	Subcutaneous.	Peri-renal.	Mesenteric.	Omental.
Inactive, calories unlimited.	44	26.2	26.4	25.4	25.2	23.2	25.3
	45	32.2	30.1	30.0	29.8	27.7	26.0
	52	26.8	26.7	26.8	27.3	24.4	26.4
	56	30.3	27.7	28.9	29.7	27.6	29.1
	59	31.6	29.8	34.9	30.3	25.5	26.7
	97	29.1	28.8	30.4	27.1	26.6	25.7
Average		29.7	28.2	29.4	28.6	25.8	26.2
Inactive, calories limited.	100	27.2	25.6	25.8	24.6	24.2	24.8
	101	27.7	27.0	27.8	26.4	24.4	25.5
	102	29.4	27.1	28.8	27.7	26.1	26.3
Average		28.1	26.9	27.5	26.2	24.9	25.5
Inactive, fasting.	98	38.8	33.3	38.8	35.4		
	99	38.8	33.0	38.7	36.6		
Average		38.8	33.2	38.8	36.0		
Active, calories unlimited, day, voluntary.	49	33.8	31.3	31.2	34.1	30.1	28.2
	50	34.4	31.6	32.0	32.4	30.8	35.3
	54	28.8	26.4	31.0	24.5	26.4	26.6
	58	33.0	32.0	32.0	31.6	29.6	29.4
Average		32.5	30.3	31.5	30.7	29.2	29.9
Active, calories limited, day, voluntary.	43	26.1	25.3	25.7	26.8	24.1	24.8
	48	34.3	31.8	33.4	34.4	30.7	34.9
	51	31.4	29.4	30.6	30.5	30.7	30.4
	53	30.0	28.3	30.2	28.3	26.7	30.7
Average		30.4	28.9	30.2	30.0	28.0	30.2
Active, calories unlimited, forced.	64	25.9	25.7	31.3	28.3	25.3	25.4
	65	27.5	27.6	29.2	27.1	27.4	27.7
	66	27.8	27.1	31.2	26.1	25.4	28.2
	93	31.6	30.7	33.1	29.6	27.8	29.6
	94	28.3	27.0	29.7	27.5	26.1	26.5
Average		28.2	27.6	30.9	27.7	26.4	27.1
Average values for all groups		31.3	29.2	31.4	29.9	29.1	27.8

The iodine number of the food fat, coconut oil, is 8.2.

(1901–02), Lummert (1898), and Schirmer (1921)). If the location of the fat does not determine its degree of saturation in all species of animals, the temperature of the region in which the fat is deposited may not be the determining factor in the degree of saturation of stored fat.

SUMMARY.[3]

The distribution of reserve fat in the female rat of 180 gm. body weight, selected as representing the end of the period of most rapid gains in weight, was approximately:

	Proportion of total fat. *per cent*
Subcutaneous	50
Genital	20
Perirenal	12
Mesenteric	10
Intermuscular	5
Omental	3

The distribution of reserve fat in a normal rat was independent of the type of diet fed; however, the amount of fat stored by a rat was greatest in rats fed a ration rich in fat.

No change in the distribution of fat was observed under any condition except forced exercise, voluntary running at night, and undernutrition. Forced activity and nocturnal activity increased the proportion of intermuscular fat. A decrease in the proportion of genital fat in the rats ingesting the starch diet followed either forced activity or undernutrition.

The degree of saturation of the stored fat was dependent upon the diet of the rat.

Fat stored in different depots showed slight variations only in degree of saturation; differences in chemical nature were not coincident with the location of the fat.

In general, the chemical nature of the fat in any one depot was not changed by activity or the amount of food eaten. The single exception was the group of rats ingesting the coconut oil diet, fasted until their body weight decreased 30 per cent.

[3] The summary of Part I will be found on p. 155.

We desire to thank The Procter and Gamble Company for liberal quantities of soy bean oil and coconut oil.

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THE RATE OF ABSORPTION OF CYSTINE FROM THE GASTROINTESTINAL TRACT OF THE WHITE RAT.

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(Received for publication, March 24, 1930.)

The amino acid cystine has recently been studied extensively in order to determine the course of its metabolism. This is of particular interest because it is an essential in nutrition and because it is one of the two known sulfur-containing amino acids derived from protein. The present study on the rate of absorption of cystine from the gastrointestinal tract of the white rat, a continuation of the series recently reported for other amino acids (1), is designed to add to the knowledge of this compound.

There is little reported in the literature to indicate how rapidly cystine is absorbed from the gastrointestinal tract. The rate of excretion of the end-products of its metabolism might indicate the relative rate of its absorption. Wolf and Österberg (2) added cystine to a basal breakfast of a man and followed the hourly excretion of total, urea, and ammonia nitrogen as well as the sulfur fractions. They observed that the increases in the nitrogen values after cystine ingestion were much less than after alanine. Unfortunately the amounts of alanine and cystine fed were very dissimilar, 50 gm. of *dl*-alanine being given and 10 gm. of cystine. In a dog (3), the results were much the same. Here the amounts fed were even more dissimilar, the quantities of alanine and cystine being 50 gm. and 3 gm., respectively. Levene and Meyer (4) administered 20 gm. of *dl*-alanine to a dog whose weight was not given and found that the total and urea nitrogen in the urine increased much more rapidly than occurred in the cystine experiment of Wolf and Österberg. There is, in this work, an indication that cystine is absorbed or metabolized less rapidly than is alanine.

More recently, Seth and Luck (5) have fed several amino acids

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to rabbits and followed the changes in the amino acid and urea nitrogen of the blood. After cystine ingestion there was a small rise in urea nitrogen and no rise in amino acid nitrogen, while several of the acids studied, particularly glycine and alanine, produced marked changes in these blood constituents. Cystine, placed in an intestinal loop of a dog, caused increases in both the urea and amino acid nitrogen of the blood, but these increments were not as great as those obtained after giving glycine or alanine. These results also indicate that cystine is absorbed more slowly than glycine or alanine. It must be remembered, however, that a rapid removal of the cystine from the blood, combined with a slow catabolism by the tissues, would give the same picture even if the absorption from the intestine were fairly rapid.

Stearns and Lewis (6) have studied the absorption of cystine (fed as the sodium salt) from the gastrointestinal tract of rabbits, using the method of Cori (7). They concluded that the compound was apparently not absorbed rapidly.

EXPERIMENTAL.

The cystine was fed as the sodium salt, or, in a few cases, as the hydrochloride, and the rate of absorption determined by the Cori technique. The rats had been grown on the stock diet recently advocated by Maynard (8) with the addition of lettuce once or twice a week. The animals weighed from 100 to 200 gm. at the conclusion of the 24 hour fasting period.

In deproteinizing the washings of the intestinal tract, the procedure was similar to that previously used (1). Filtration after heat coagulation was not attempted as it was felt that there might be a loss of some of the cystine at the reaction required for the coagulation. The coagulated protein was filtered out of the solution along with the precipitate obtained with tungstic acid.

The colorimetric method of Folin and Marenzi (9) was used for the determination of cystine. Recovery of cystine added to intestinal washings was within the limits of error of this method.

In a preliminary series of experiments the residual cystine in the gastrointestinal tract of rats which had been fasted 24 hours was determined. That the color developed was not entirely due to cystine was shown by the fact that a certain amount of color was obtained when the phosphotungstic acid reagent was added with-

out the preliminary reduction with sulfite. Regardless of what these reducing substances might have been, they produced a color in a cystine determination and were calculated as cystine. The color developed by the intestinal washings of the control rats was too faint to compare with the standard. It was necessary, therefore, to add a known amount of cystine before making the determination. The residual cystine values are given in Table I. With a few exceptions, the values, calculated as mg. of cystine per 100 gm. of body weight, are fairly uniform.

TABLE I.

Reducing Substances in Intestine of Fasting Rats. Calculated as Cystine.

Rat No.	Sex.	Weight.	Cystine per 100 gm. body weight.
		gm.	*mg.*
1	F.	114	19.3
2	M.	176	14.2
3	"	145	20.7
4	"	138	15.2
5	F.	105	10.5
6	"	99	21.2
7	"	122	16.4
8	"	111	11.7
17	"	152	25.6
18	"	183	30.0
19	"	157	19.1
20	"	155	19.4
Average			18.6

It was considered important to determine whether the cystine was altered to any appreciable extent by bacterial action. Cori (7) decided that the destruction of glucose by bacteria was neglible when he found that the incubation of a gastrointestinal tract containing this sugar did not lead to a decrease in the amount of the sugar. For cystine this method was not applicable. The amino acid would have to be introduced as an acid or basic salt, either of which would have distinctly altered the reaction of the intestine and probably, therefore, the bacterial action. While the cystine was fed to the rats as an acid or basic salt, the conditions were not similar, because in the living animal there would be opportunity

for neutralization and for absorption of the acid or base. A method which might be considered as indicative of the amount of bacterial action was tried. Rats were fed cystine (as the sodium salt) or water, and 2 hours later were killed, the intestinal contents being worked up as usual. The intensities of the colors developed when these solutions were treated with the phospho-

TABLE II.

Rate of Absorption of Cystine Fed as Sodium Salt.

Rat No.	Sex.	Weight after fast.	Absorption time.	Cystine fed, per 100 gm. body weight.	Cystine recovered, per 100 gm. body weight.	Rate of absorption, per 100 gm. body weight per hr.
		gm.	*hrs.*	*mg.*	*mg.*	*mg.*
9	F.	171	2	128	70	29
10	"	170	2	129	62	33
25	"	170	2	145	79	33
27	"	168	2	146	89	29
Average						31.0
11	F.	160	3	137	44	31
26	"	153	3	161	80	27
28	"	155	3	158	70	29
29	"	191	3	129	36	31
40	"	157	3	161	65	32
Average						30.0
31	F.	143	4	173	40	33
32	"	156	4	148	36	28
33	M.	147	4	163	49	29
37	"	140	4	170	40	32
Average						30.5
" of all experiments						30.5

tungstic acid reagent without the preliminary addition of sulfite, were compared. The solutions from the intestines of the cystine-fed rats developed about twice as much color as those from the controls. This extra reducing material might be cysteine or it might be mercaptans or sulfides. It has been neglected, however, since in actual amount it was so small, the amount present at the end of 2 hours being estimated to be equivalent to 2 to 3 mg. of

cystine. It is realized that this method of approach indicates nothing in regard to that cystine which may have been altered and then absorbed before the death of the animal.

In Table II the rate of absorption of cystine fed as the sodium salt is recorded. The individual determinations are very uniform, and, as has been shown previously for certain other substances (1, 7), these rates are not affected by the length of time of absorption and the resulting changes in the amount of cystine left in the tract. Several cases with possible diarrhea are not included in Table II although some of them had values within the limits given.

When the cystine was fed as the hydrochloride, the results were quite different. Only four rats were used. The rates of absorption were 9, 9.5, 8, and 12 mg. of cystine per 100 gm. per hour. The upper part of the small intestine was white with what appeared to be a coating of precipitated cystine in two of the four rats. For this reason the results were not considered to be of any particular value and the experiment was not repeated. Two later attempts to get this white coating failed although the intestinal contents of these rats did contain hexagonal crystals of cystine. A microscopic examination of the intestines of two rats fed the sodium salt showed cystine crystals in one.

DISCUSSION.

The values obtained in this series of experiments indicate that cystine is absorbed much more slowly than any of the other amino acids which have been studied so far. It is again demonstrated that a study of the nitrogen fractions of blood and urine gives a fair indication of the relative rates of absorption of amino acids.

From the data of Stearns and Lewis (6), the rate of absorption of cystine by rabbits has been calculated. The values obtained (10.8 and 12.7 mg. of cystine per 100 gm. of body weight per hour) are about one-third of those found for rats. If the absorption rates are calculated on a basis of a unit of body surface rather than on body weight, the following values result: rabbits, 13.5 and 14.1 mg.; rats, 17.9 mg. of cystine per 100 sq. cm. of body surface per hour. It is clear that the agreement is much better when the calculations are made in this way. Cori and Cori (10) found that the rate of absorption of glucose, calculated on the basis of a unit

of body weight, was about 2.5 times as great for mice as for rats, and they suggested that the rate of absorption may be more or less directly proportional to the metabolism. These cystine figures tend to substantiate this suggestion.

SUMMARY.

The rate of absorption of cystine, fed as the sodium salt, from the gastrointestinal tract of the rat, has been determined. The absorption is at the rate of 30.5 mg. of cystine per 100 gm. of body weight per hour, a value lower than that of any other amino acid which has been reported. When fed as the hydrochloride, it is absorbed still more slowly, which may be due to an apparently decreased solubility of the cystine.

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BLOOD AS A PHYSICOCHEMICAL SYSTEM.

IX. THE CARBON DIOXIDE DISSOCIATION CURVES OF OXYGENATED HUMAN BLOOD.

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The preceding papers of this series describe the physicochemical properties of several normal and pathological specimens of blood. It is now our purpose to commence the descriptive study of human blood in general, an undertaking which involves an increase in the number of independent variables of the system. Hitherto the independent variables have been two, oxygen pressure and carbon dioxide pressure; in order to accomplish our present object they must in the end be increased to not less than eight (1). It seems probable that the task may best begin with a comparative study of carbon dioxide dissociation curves ((1) p. 358).

Peters, Bulger, and Eisenman (2) have set up the following equation as a description of the carbon dioxide dissociation curves of oxygenated human blood:

$$\Delta = 0.334 \text{ Hb} + 2.83 \tag{1}$$

Here Δ represents the difference between the total carbonic acid content, expressed in millimols per liter, of oxygenated human blood at a pressure of carbon dioxide of 60 mm. and the content at a pressure of carbon dioxide of 30 mm. The quantity Hb represents the concentration of hemoglobin expressed as millimols of oxygen capacity per liter of blood. The equation is based upon a study of 51 specimens of human blood. It fits the measurements well, one-half the errors of Δ falling within the range ± 0.25 mM.

During the past 4 years we have accumulated 117 similar measurements which are given in Columns 1 and 5 of Table I. If these data are fitted to Equation 1 one-half the errors fall within

TABLE I.

Oxygen capacity.	Total CO_2 at pCO_2.			Δ (total $CO_2)_{60}$ − (total $CO_2)_{40}$.		Error (chart − observed).
	30 mm.	40 mm.	60 mm	Observed.	Chart.	
(1)	(2)	(3)	(4)	(5)	(6)	(7)
mM per l.	*mM per l.*	*mM per l.*	*mM per l.*	*mM per l.*	*mM per l.*	*mM per l.*
10.11	15.41	17.16	20.07	4.66	5.78	+1.12
8.74	18.59	19.90	21.97	3.38	5.68	+2.30
9.62	19.41	21.70	25.47	6.06	6.24	+0.18
10.29	17.12	19.54	23.59	6.47	6.19	−0.28
9.80	17.67	20.10	23.95	6.28	6.09	−0.19
10.88	14.56	16.83	20.58	6.02	5.97	−0.05
7.65	18.92	21.08	24.34	5.42	5.43	+0.01
8.58	16.45	18.64	22.43	5.98	5.47	−0.51
9.83	16.88	19.32	23.36	6.48	5.99	−0.49
8.79	18.56	20.86	24.48	5.92	5.81	−0.11
9.96	15.86	18.16	21.93	6.07	5.87	−0.20
10.38	17.25	19.65	23.41	6.16	6.23	+0.07
10.56	16.84	19.20	22.87	6.03	6.24	+0.21
8.68	19.15	21.50	25.17	6.02	5.87	−0.15
9.41	19.15	21.50	25.15	6.00	6.13	+0.13
10.08	17.53	20.00	23.96	6.43	6.17	−0.26
8.42	16.86	19.05	22.65	5.79	5.48	−0.31
8.26	19.20	21.22	24.27	5.07	5.66	+0.59
8.82	16.28	18.53	22.12	5.84	5.54	−0.30
9.51	18.42	20.76	24.53	6.61	6.07	−0.54
10.27	17.66	20.13	24.16	6.50	6.27	−0.23
9.27	18.60	20.76	24.18	5.58	5.99	+0.41
9.98	16.21	18.60	22.56	6.35	5.54	−0.81
9.45	17.07	19.36	23.14	6.07	5.85	−0.22
10.13	15.63	17.80	21.34	5.71	5.87	+0.16
8.78	20.12	22.41	26.14	6.02	6.01	−0.01
8.86	18.60	20.99	24.67	6.07	5.85	−0.22
8.92	19.55	26.88	25.57	6.02	6.00	−0.02
8.07	18.96	22.00	25.15	6.19	5.78	−0.41
9.11	17.43	19.72	23.45	6.02	5.79	−0.23
9.33	18.52	20.85	24.67	6.15	6.02	−0.13
9.86	18.02	20.36	24.17	6.15	6.14	−0.01
9.13	19.37	21.48	24.77	5.40	5.94	+0.54
9.28	13.62	15.51	18.65	5.03	5.34	+0.31
8.63	19.45	21.57	24.84	5.39	5.68	+0.29
9.29	16.13	18.30	21.92	5.79	5.68	−0.11
9.89	17.84	20.18	24.00	6.16	6.12	−0.04
10.09	17.26	19.50	23.10	5.84	6.10	+0.26
8.91	18.88	21.17	24.71	5.83	5.76	−0.07

TABLE I—*Continued.*

Oxygen capacity.	Total CO_2 at pCO_2.			Δ (total $CO_2)_{60}$ − (total $CO_2)_{40}$.		Error (chart − observed).
	30 mm.	40 mm.	60 mm.	Observed.	Chart.	
(1)	(2)	(3)	(4)	(5)	(6)	(7)
mM per l.	*mM per l.*	*mM per l.*	*mM per l.*	*mM per l.*	*mM per l.*	*mM per l.*
9.40	15.41	17.57	21.12	5.71	5.61	−0.10
8.92	18.66	20.90	24.50	5.84	5.85	+0.01
9.93	16.54	18.83	22.55	6.01	5 98	−0.03
9.48	18.47	20.80	24.62	6.15	6.06	−0.09
10.51	16.35	18.70	22.47	6.12	6.14	+0.02
9.37	17.39	19.76	23.64	6.25	5.88	−0.37
9.60	17.88	20.13	23.68	5.80	6.01	+0.21
8.82	18.97	21.43	25.28	6.31	5.91	−0.40
9.70	17.30	19.45	23.06	5.76	5.97	+0.21
9.04	19.32	21.57	25.08	5.76	5.99	+0.23
9.70	16.18	18.08	21.30	5.12	5.84	+0.72
9.11	15.86	17.65	20.63	4.77	5.53	+0.76
9.62	11.37	13.21	16.31	4.94	5.09	+0.15
9.92	12.54	14.78	18.66	6.12	5.37	−0.75
8.84	13.34	15.36	18.82	5.48	5.13	−0.35
8.28	17.52	19.90	23.82	6.30	5.51	−0.79
7.78	24.99	27.13	30.57	5.58	6.24	+0.66
8.71	21.60	23.81	27.18	5.58	6.18	+0.60
9.01	19.40	21.74	25.47	6.07	6.02	−0.05
9.56	17.70	20.08	23.86	6.16	5.99	−0.17
9.08	19.76	21.83	25.11	5.35	6.06	+0.71
10.49	14.73	16.98	20.71	5.98	5.87	+0.11
10.02	10.24	12.04	15.09	4.85	4.92	+0.07
10.80	8.58	10.20	13.07	4.49	4.56	+0.07
7.86	22.72	25.16	28.84	6.12	6.01	−0.11
8.77	18.91	21.30	25.03	6.12	5.85	+0.27
6.22	21.34	23.36	26.92	5.58	5.14	−0.44
6.01	22.91	25.10	28.35	5.54	5.30	−0.14
5.96	21.25	23.18	26.60	5.35	5.07	−0.28
7.13	20.20	22.32	25.60	5.40	5.38	−0.02
6.16	19.68	21.57	24.44	4.76	4.99	+0.23
5.32	19.77	21.70	24.72	4.95	4.79	−0.16
6.34	18.48	20.67	23.60	5.12	5.00	−0.12
7.24	18.82	20.84	24.17	5.35	5.29	−0.06
6.77	20.20	22.42	25.86	5.66	5.29	−0.37
7.98	19.86	21.97	25.16	5.30	5.65	+0.35
8.55	22.00	24.34	28.20	6.20	6.16	−0.04
8.45	18.54	20.68	24.02	5.48	5.67	+0.19
8.12	18.40	20.40	23.62	5.22	5.51	+0.29

TABLE I—*Concluded.*

Oxygen capacity.	Total CO_2 at pCO_2.			Δ (total $CO_2)_{60}$ − (total $CO_2)_{40}$.		Error (chart − observed).
	30 mm.	40 mm.	60 mm.	Observed.	Chart.	
(1)	(2)	(3)	(4)	(5)	(6)	(7)
mM per l.	*mM per l.*	*mM per l.*	*mM per l.*	*mM per l.*	*mM per l.*	*mM per l.*
9.60	18.56	20.80	24.33	5.77	6.10	+0.33
6.82	23.72	25.61	28.66	4.94	5.68	+0.74
6.60	22.90	25.02	28.20	5.30	5.50	+0.20
5.50	22.05	23.95	27.00	4.95	4.99	+0.04
6.61	22.01	24.08	27.41	5.40	5.41	+0.01
7.36	19.18	21.11	24.22	5.04	5.35	+0.31
8.57	20.92	23.22	26.94	6.02	6.03	+0.01
7.69	20.08	22.15	25.52	5.44	5.56	+0.12
7.60	19.40	21.57	24.98	5.58	5.47	−0.11
8.79	19.10	21.35	24.90	5.80	5.91	+0.11
4.00	22.47	24.26	27.10	4.63	4.56	−0.07
5.98	23.40	25.98	29.92	6.52	5.37	−1.15
5.40	20.27	22.14	25.17	4.90	4.84	−0.06
5.29	22.23	24.12	27.04	4.81	4.92	+0.11
5.90	19.74	21.88	25.24	5.50	4.96	−0.54
5.97	21.73	23.62	26.54	4.81	5.11	+0.30
7.14	21.11	23.10	26.51	5.40	5.47	+0.07
6.72	21.26	23.36	26.72	5.46	5.38	−0.08
7.12	19.63	21.70	25.07	5.44	5.34	−0.10
6.12	20.13	22.19	25.48	5.35	5.04	−0.31
3.20	7.28	8.32	10.02	2.74	3.34	+0.60
2.50	5.71	6.74	8.54	2.83	2.88	+0.05
3.77	8.63	10.06	12.58	3.95	3.75	−0.20
9.82	6.50	7.82	10.12	3.62	3.75	+0.13
3.75	21.57	23.38	26.07	4.50	4.44	−0.06
12.70	14.92	17.48	21.63	6.71	6.68	−0.03
10.50	5.91	7.19	9.46	3.55	3.55	0.00
3.08	13.93	15.46	17.97	4.04	3.91	−0.13
4.57	12.18	13.93	16.85	4.67	4.24	−0.43
10.51	8.99	10.76	13.85	4.86	4.68	−0.18
12.00	16.60	19.10	23.32	6.72	6.77	+0.05
4.44	22.42	24.17	26.92	4.50	4.68	+0.18
6.26	19.77	21.79	24.98	5.21	5.06	−0.15
7.09	17.21	19.27	22.60	5.39	5.11	−0.28
8.76	13.70	15.77	19.18	5.48	5.25	−0.23
5.36	15.41	17.17	19.95	4.54	4.58	+0.04
6.14	13.66	15.46	18.42	4.76	4.68	−0.08
7.95	12.04	13.94	17.08	5.04	4.90	−0.14
8.82	9.26	11.00	14.02	4.76	4.62	−0.14

the range ± 0.30 mM. This small increase in the probable error is due to the presence in our studies of a considerable number of specimens of blood in which the level of the carbon dioxide dissociation curves was either very high or very low. In such instances, especially when the value of Hb is high, the errors are large and systematic.

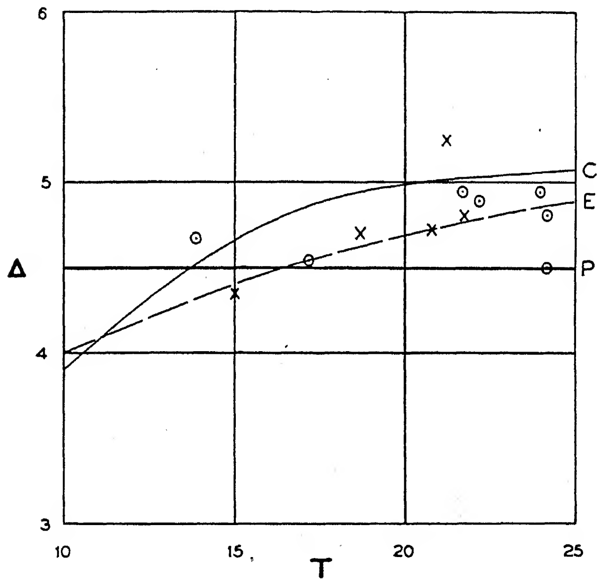

FIG. 1. Values of Δ as a function of T_{40} when Hb = 5.0 mM ± 0.5, × = Peters' observations, ⊙ = our observations, P = Peters' approximation, E = our approximation, C = calculated relation. Δ and T are measured in mM per liter of blood.

The nature of the case may be illustrated by means of Figs. 1 and 2. Fig. 1 shows for all cases where 4.5 mM > Hb > 5.5 mM the values of Δ as a function of T_{40} (*i.e.*, mM of total carbonic acid per liter of blood at a pressure of 40 mm. of CO_2). Fig. 2 shows the same relation for all cases where 10.0 > Hb > 11.0. On each of these figures straight lines are drawn corresponding to Equation 1, and also two curves. The broken curves are taken from a contour line chart (Fig. 3 below) which has been empirically fitted to our data, taking account of the three variables Hb, Δ, and T_{40}.

The continuous curves are taken from a similar chart obtained by calculation as explained below. Figs. 1 and 2 show that when Hb is constant Δ varies with T_{40}. It varies largely in the neighborhood of Hb = 10 mM, narrowly in the neighborhood of Hb = 5 mM.

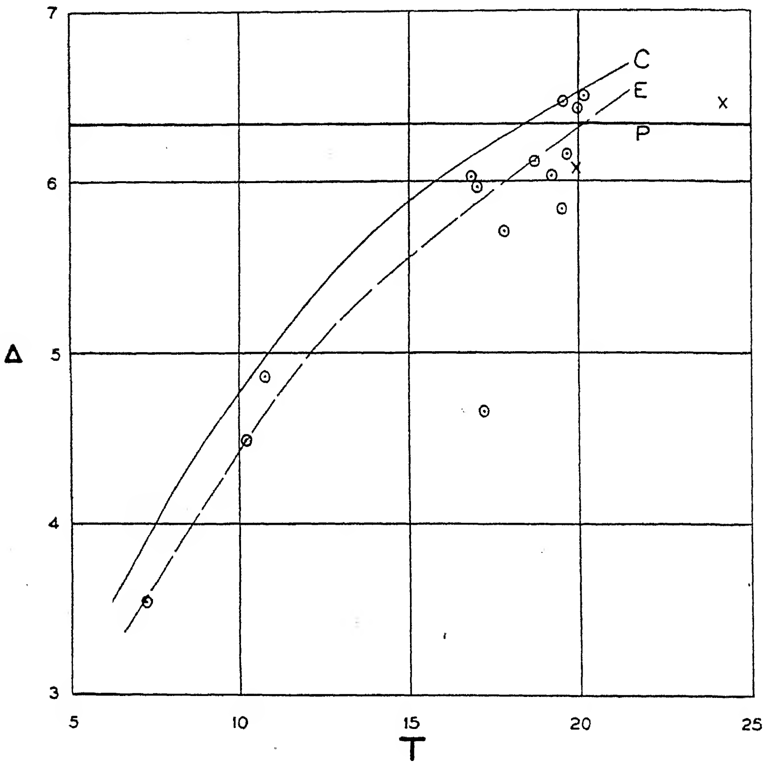

Fig. 2. Values of Δ as a function of T_{40} when Hb = 10.5 mM ± 0.5, $\times$ = Peters' observations, $\odot$ = our observations, P = Peters' approximation, E = our approximation, C = calculated relation. Δ and T are measured in mM per liter of blood.

Fig. 3, as just stated, is a chart fitted empirically to our data. It was completed before any theoretical calculations had been made and without any independent knowledge concerning its probable form. In the construction Peters' data were not considered, but were kept as a check on the validity of the result.

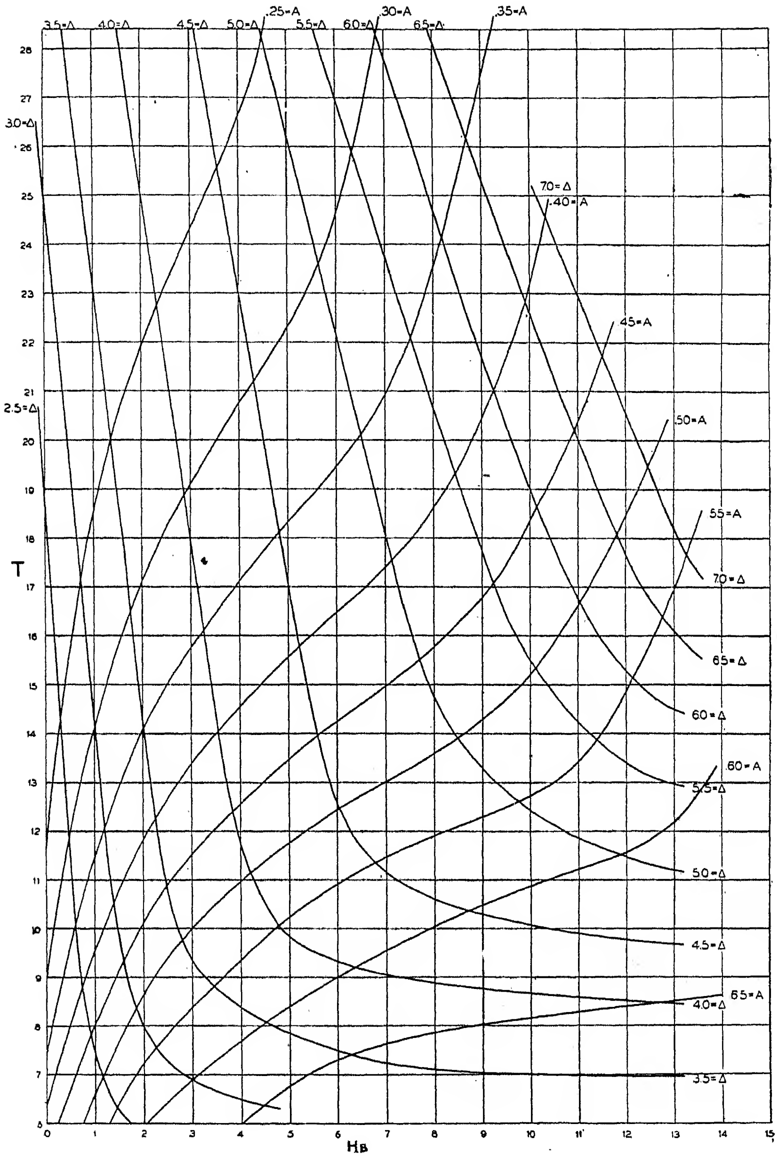

FIG. 3. Cartesian nomogram showing Δ and A as functions of Hb and T_{40}. T and Hb are measured in mM per liter of blood.

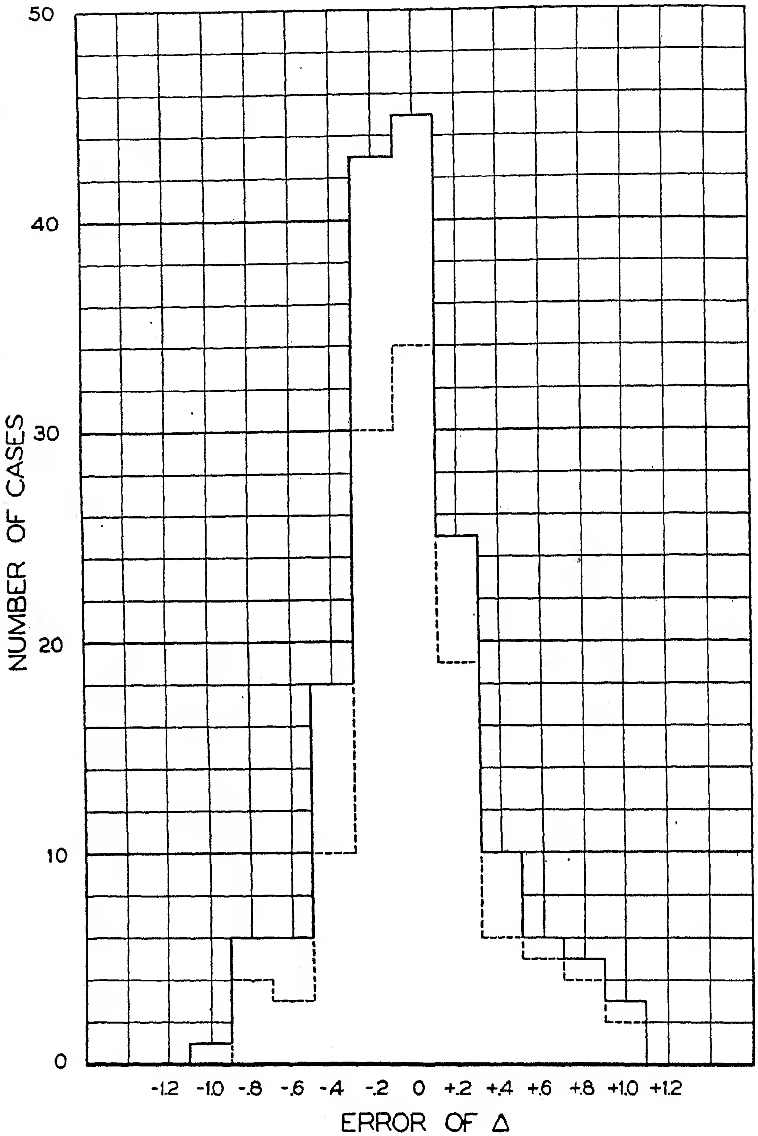

Fig. 4. Distribution of errors in fitting data to Fig. 3. Broken lines indicate our data; continuous lines, all data. Δ is measured in mM per liter of blood.

On the figure, values of Hb are expressed as abscissas, values of T_{40} as ordinates, values of Δ as contour lines. Another family of contour lines shows the values of A, the slope of the graph of the function $\log T = A \log (pCO_2) + B$. It will be remembered that the logarithmic graphs of carbon dioxide dissociation curves are approximately straight lines.

All necessary information concerning the fit of the chart to the 117 experimental points is given in Table I. One-half the errors of Δ fall within the range ±0.20 mM.

Peters' data fit our chart (Fig. 3) with errors of Δ (chart minus observed values) one-half of which fall within the range ±0.25 mM. But in this case there is also a mean error of -0.10, which may perhaps be attributed to a small systematic difference between the results of his laboratory and of ours. If this fact be taken into account one-half the errors again fall within the range ±0.20 mM.

Since the errors involved in our approximations must be taken as a basis for an estimate of the magnitude of the effects that are due to the neglect of other variables, the distribution of errors in our data (broken lines) and in all data (Peters' and ours) is shown as a histogram on Fig. 4. On this figure the five large errors have for convenience been included in the compartments designated $+1.0$ and -1.0. Among these errors a few are probably due to gross experimental inaccuracies. In the main, however, they may be attributed to variations in the concentrations of water, serum protein, and other substances. Fig. 4 clearly demonstrates the slight systematic discrepancy between Peters' data and ours.

If we assume, for a given value of pH_s, say 7.4, constant concentration of protein in serum and constant concentration of hemoglobin in red cells, it is possible to construct a chart similar to Fig. 3 from our knowledge of the properties of the components of the system.

Let
x = mM bicarbonate per liter blood
x_s = " serum bicarbonate per liter blood
x_c = " cell " " " "
v_s = liters serum volume per liter blood
v_c = " cell " " " "
$v_c = 0.05 \cdot Hb$
70 = gm. protein per liter serum

$BHbO_2$ = mM base combined with oxyhemoglobin per liter blood
BP_s = " " " " serum protein " " "
$B = x + BHbO_2 + BP_s$
$pH_c = 0.75\ pH_s + 1.5$

Then
$$x_s = \frac{7.95 \times 10^{-7}}{(H)_s} \times 0.031 \times pCO_2 \times (1 - 0.05\ Hb)$$
$$x_c = \frac{11.75 \times 10^{-7}}{(H)_c} \times 0.0264 \times pCO_2 \times 0.05\ Hb$$
and
$$\frac{x}{pCO_2} = \frac{0.2464 \times 10^{-7}}{(H)_s} - \frac{0.01232 \times 10^{-7}}{(H)_s} \times Hb\ \frac{0.4904 \times 10^{-7}}{(H)_s^{0.75}} \times Hb \quad (2)$$
Also $BHbO_2 = 3.6\ Hb\ (pH_c - 6.41)$
$$BP_s = 0.104 \times 70(1 - 0.05\ Hb)\ (pH_s - 5.08)$$
and $$B - x = (2.336\ pH_s - 15.83)Hb + 7.28\ pH_s - 36.98 \quad (3)$$

From Equations 2 and 3 $(\overset{+}{H})_s$ and pH_s may be eliminated and the values of x for pressures of carbon dioxide of 30, 40, and 60 mm. may be obtained as functions of B and Hb, corrections being applied for changes in cell volume with changes in pH_s. These results yield values of T_{40}, T_{30}, T_{60}, and Δ. These values of Δ are shown as contour lines on Fig. 5. For comparison the corresponding contour lines of Fig. 3 are represented as broken lines. The agreement between the empirically determined and the theoretically calculated values is satisfactory. A systematic discrepancy between the two families of contour lines for a part of Fig. 5 is no doubt due to errors in the assumed concentrations of protein or to errors in the numerous constants employed in setting up Equations 2 and 3.

On Fig. 6 values of B as a function of Hb and T_{40} are given. This figure is of course affected by the small errors just described. The value of B is here that above defined; *viz.*, the base distributed between carbonic acid and protein.

The results expressed by Fig. 3 may be employed as a means of determining the carbon dioxide dissociation curve of a specimen of oxygenated blood when the oxygen capacity and the value of T for a single pressure of carbon dioxide are known. This is done most conveniently with the aid of Fig. 7.

The procedure may be illustrated by means of an example. Given the data:

$$Hb = 10.56\ \text{mM}$$
$$P = 65.0\ \text{mm.}$$
$$T_{65} = 23.7\ \text{mM}$$

First, a straight line is passed on Fig. 7 between the point 65.0 on the scale P and the point 23.7 on the scale T.

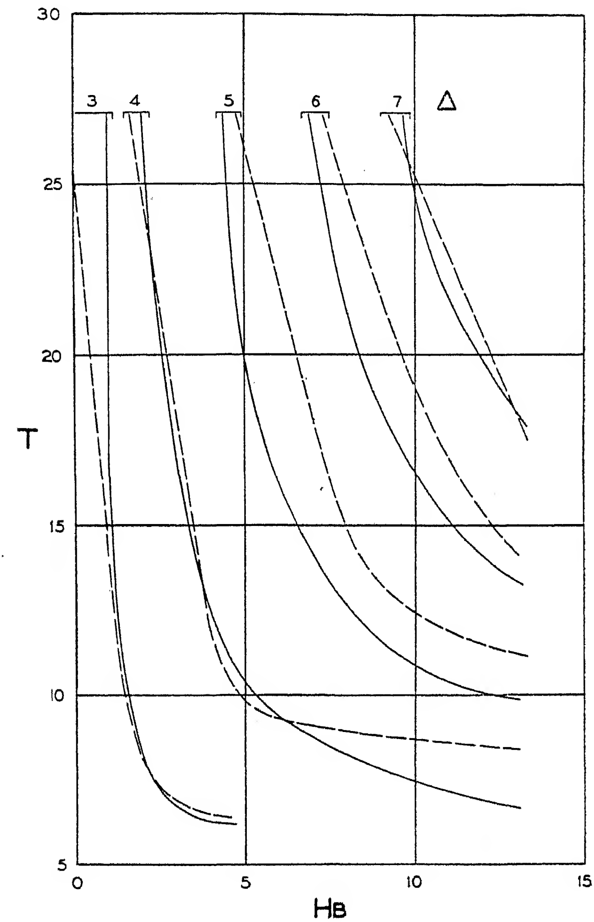

FIG. 5. Cartesian nomogram showing Δ as a function of Hb and T. Continuous lines are calculated; broken lines, empirically fitted. T, Hb, and Δ are measured in mM per liter of blood.

Secondly, the point of intersection of this line with the curve corresponding to Hb = 10.56 (estimated by interpolation between the curves for Hb = 10 and Hb = 11) is marked.

Thirdly, pairs of intercepts on the scales T and P of straight lines through this point are read. Each of these pairs of values

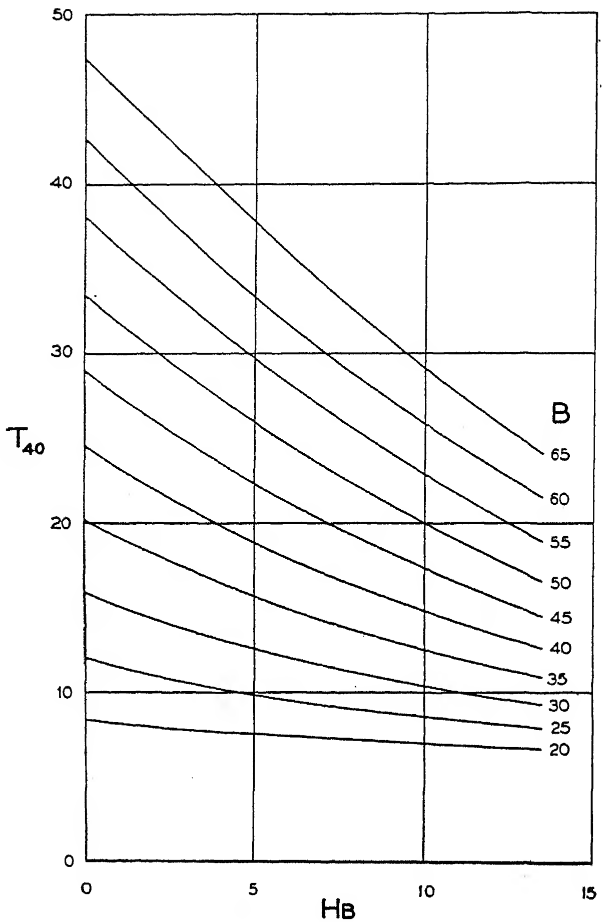

FIG. 6. Cartesian nomogram showing $B = BHCO_3 + BHbO_2 + BP_s$ as a function of Hb and T_{40}. T, Hb, and B are measured in mM per liter of blood.

is a pair of coordinates of a point on the desired carbon dioxide dissociation curve. For example, in this instance when $P = 40.0$, $T_{40} = 19.1$ and this reading may be compared with the experi-

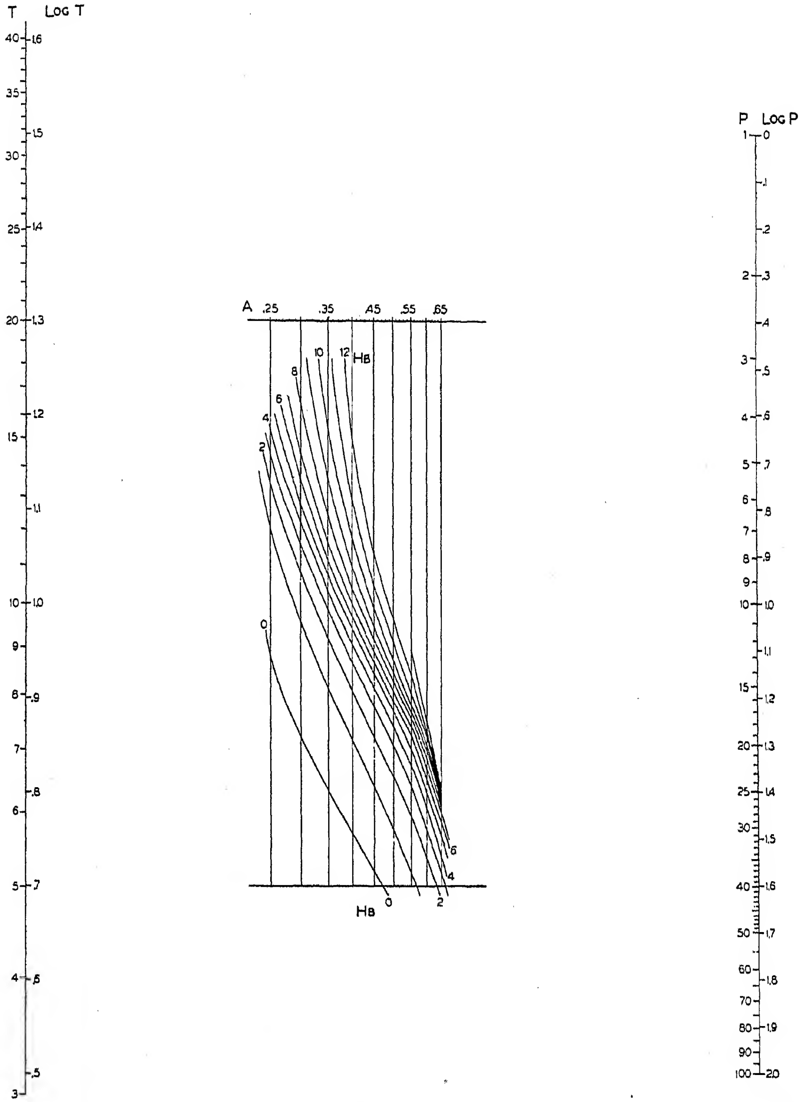

FIG. 7. Alignment chart showing relation between T, P, Hb, and A.

mentally determined value $T_{40} = 19.2$. If the dissociation curve itself is desired the points (1) $P = 65.0$, $T = 23.7$ and (2) $P = 40.0$, $T = 19.1$ may be plotted logarithmically and joined by a straight line. It is evident, however, that Fig. 7 is itself a nomographic representation of all the carbon dioxide dissociation curves that can be derived from it. It is affected by the errors above discussed as well as those dependent upon the assumption that log T is a linear function of log P.

TABLE II.

Values of Log T_{40} (from Fig. 3).

Hb	A								
	0.25	0.30	0.35	0.40	0.45	0.50	0.55	0.60	0.65
mM									
0	1.066	0.954	0.875	0.803					
1	1.268	1.149	1.057	0.980	0.903	0.820			
2	1.340	1.233	1.149	1.072	1.004	0.935	0.857	0.771	
3	1.386	1.281	1.199	1.127	1.061	1.000	0.924	0.839	
4	1.425	1.317	1.233	1.163	1.100	1.039	0.971	0.887	0.778
5		1.348	1.263	1.193	1.129	1.072	1.009	0.924	0.829
6		1.391	1.289	1.216	1.154	1.093	1.037	0.954	0.863
7			1.320	1.241	1.176	1.114	1.059	0.980	0.884
8			1.371	1.270	1.199	1.134	1.076	1.002	0.895
9			1.438	1.310	1.225	1.154	1.090	1.019	0.903
10				1.364	1.264	1.182	1.104	1.035	0.914
11					1.310	1.220	1.127	1.051	0.919
12						1.267	1.172	1.063	0.924
13							1.228	1.086	0.929

By means of Fig. 6 it is also possible to determine roughly the value of B and therefore the amount of base bound by protein. Thus for $T_{40} = 19.1$ and Hb = 10.56 we may read on Fig. 6, B = 49 mM per liter. Since at a pressure of 40 mm. of carbon dioxide there are present about 1.15 mM of free carbonic acid per liter of blood it follows that approximately 19 mM of base per liter are associated with carbonic acid and about 30 mM per liter with the proteins.

The scale of Fig. 6 is too small for the highest attainable accuracy. Therefore it seems desirable to give a description of its recon-

struction on a sufficiently large scale, say on a sheet of coordinate paper 43 cm. × 56 cm. in size, such as we have used.

First the extreme left-hand side of the sheet is graduated from below upward in values of log T, taking 50 cm. as the unit of length and placing the value log T = 1.0 near the middle of the scale. On this scale that of T is superimposed.

Secondly, the extreme right-hand side of the sheet is graduated from above downward in values of log P, taking 25 cm. as the unit of length and placing the value log P = 1.0 on the ordinate of the sheet already marked by the point log T = 1.0. On this scale that of P is superimposed.

Thirdly, calling the horizontal distance between the two scales 1.00 and measuring from left to right, vertical lines are erected at distances 0.3333, 0.3750, 0.4118, 0.4444, 0.4737, 0.5000, 0.5238, 0.5455, and 0.5652 from the left-hand scale. These lines are marked A 0.25, 0.30, 0.35,. . .0.65.

The figure is now prepared for the construction of the curves of hemoglobin. For this purpose Table II, obtained from Fig. 3, is employed.

Table II gives for P = 40 (*i.e.*, log P = 1.602) simultaneous values of log T, A, and Hb. For example, the first entry of Table II is to be read as follows:

(P = 40)	log P = 1.602
(T_{40} = 11.65)	log T_{40} = 1.066
	A = 0.25
	Hb = 0.00

Accordingly a line is passed through the point 1.602 of the scale of log P and the point 1.066 of the scale of log T, and its intersection with the line A = 0.25 is marked. This process is repeated for the other entries of the first row of Table II; a smooth curve is drawn through the marked points and the curve is designated Hb = 0. Similarly the other rows of the table permit the construction of the curves for values of Hb from 1 to 13 inclusive.

A figure thus constructed will with care give trustworthy results which are liable only to such errors as are present in Table I.

SUMMARY.

The results of Peters, Bulger, and Eisenman concerning the relation between oxygen capacity of human blood and the carbon dioxide dissociation curve of oxygenated blood are confirmed.

It is shown that this relation is relatively inaccurate for high and low ranges of the values of T_{40} (total CO_2 when $pCO_2 = 40$ mm.).

A contour line chart is given defining the properties of the dissociation curve as functions of the two variables, oxygen capacity and T_{40}. The use of this chart is affected by small errors which may be provisionally ascribed to neglect of other variables such as the concentration of hemoglobin in the red blood cells and of protein in the plasma.

A method is given for constructing the carbon dioxide dissociation curve of a specimen of oxygenated blood by means of the determination of (1) the total carbonic acid content at any pressure of carbon dioxide and (2) the oxygen capacity of the specimen.

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THE PHYSICAL CHEMISTRY OF THE PROTEINS IN NON-AQUEOUS AND MIXED SOLVENTS.*

I. THE STATE OF AGGREGATION OF CERTAIN PROTEINS IN UREA-WATER SOLUTIONS.

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The use of solvents, other than water, that are capable of dissolving proteins without entering into chemical combination with them, in contrast to the usual mode of procedure, by the addition of small amounts of acid or alkali, makes it possible to investigate the properties of proteins in one of their most important states, the isoelectric state. In the present investigation, one such property, the osmotic pressure, was measured chiefly, with the use of urea-water mixtures as a solvent. The main objectives in mind were: (1) to develop a method for the determination of molecular weights of proteins that are not soluble in water in the isoelectric state, and (2) to determine whether proteins are capable of undergoing changes in their state of aggregation with changes of solvent.

Neutral solvents other than water have been little used in connection with protein studies. Cooper and Nicholas (1) find that, in general, proteins are insoluble in organic solvents. It has long been known that mixtures of alcohol and water dissolve a class of proteins known as the prolamines, which do not dissolve in either of the pure components. This mixed solvent is, however, specific for the prolamines. A few organic solvents are known to bring into solution, without entering into chemical combination, some of the most insoluble proteins. Urea, for example, when dissolved in a small amount of water has a powerful solvent action on many proteins, irrespective of their class. Besides urea solu-

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tions, liquid phenol, formamide, urethane, and thiourea solutions behave similarly, but not quite so efficiently toward proteins. Spiro (2), in 1900, was the first to report the solvent action of urea solutions for proteins. Dill and Alsberg (3) have made use of urea in the study of the prolamine, gliadin. Urea solution is also known to have a powerful solvent action on starch.

The selection of organic solvents in which to investigate certain properties of proteins, in contrast to the usual mode of investigation, *i.e.* in pure water in acid or alkaline solutions, seems desirable for a number of reasons. First, only relatively few proteins are soluble in pure water or dilute salt solutions, thus setting limits to any comparative or extensive study; second, the use of small amounts of acid or alkali as is customarily done, to bring the protein into solution, gives rise to complicating factors resulting from a chemical combination of the protein with acid or alkali, and masks such properties of the protein as aggregation, which otherwise might be noticeable, if the protein could be dissolved in the uncombined state; and third, it frequently happens that the use of solvents other than water brings out results that are entirely obscured in aqueous solution (4).

EXPERIMENTAL.

In most of the experiments a 6.66 molar urea solution was used as solvent. The type of osmometer used for determining the osmotic pressure of a protein solution is shown in Fig. 1. The outer vessels were measuring cylinders about 40 cm. in height. The collodion bag, containing the protein solution, is securely bound to the rubber stopper at the lower end of the manometer tube by means of rubber string. Cut rubber bands and the roll of rubber from the inside of a golf ball were used. The bag and manometer tube are held suspended in place in the cylinder containing the outer protein-free solution by means of a rubber stopper inserted at the top. The system is kept free from carbon dioxide, ammonia, and other gaseous impurities in the air by a rubber connection joining the spaces above the inner and outer solutions. The pressure of these spaces is kept equal and atmospheric by opening this connection to the air for a few seconds from time to time during the experiment.

With this type of osmometer, a number of factors was found to

influence the rate of attainment of equilibrium. When large, broad, collodion bags were used, and the osmometer was allowed simply to stand at 0°, equilibrium was reached very slowly (2 to 3 months). To attain a more rapid approach of equilibrium, the bags were made long and narrow in shape to give as great a sur-

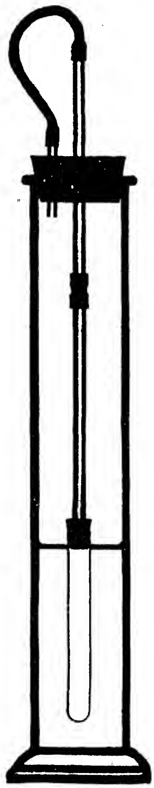

Fig. 1. Apparatus for determining the osmotic pressure of protein solutions. The mechanical rocking arrangement is not shown.

face per unit of volume of the solution as was feasible. They were made of such size as to hold an amount of protein solution sufficient for duplicate analyses and pH determination, in most cases of about 15 cc. capacity. The volume of the outer solutions was about 200 cc.

The rate of attainment of equilibrium was increased by stirring. To do this, the osmometers were placed on a framework rack, holding three cylinders on each side, resting on a central pivot bar, and rotated back and forth by means of an eccentric and motor. The motor was geared down to produce slow motion, which was maintained continuously during the adjustment of the solution in the manometer tube to the equilibrium value. The actual stirring of the solutions was chiefly accomplished by having the lower portion of the manometer tube, to which the bag was attached, act like a pendulum. This was accomplished, as shown in Fig. 1, by breaking the manometer tube and rejoining by a small piece of rubber tubing. The apparent motion of the bag from one side to the other served to stir both inner and outer solutions. In this way the time of reaching equilibrium was reduced from months to a period of less than 2 weeks.

After some preliminary trials, Schering's celloidin[1] was chosen for use, because bags made from solutions of this collodion gave, without any treatment, a better membrane than those prepared from other collodion solutions tried by us.

The membranes were prepared from a solution of 7 per cent collodion, prepared by dissolving the shreds of Schering's celloidin in 40 volumes per cent of absolute alcohol and 60 volumes per cent of absolute ether. They were cast from the inside of small test-tubes. (If the tubes are unused and have a well glazed surface there is never any difficulty in removing the membrane from the inside of the tubes.) During the period of drying, the test-tubes were rotated continually to insure uniformity of thickness. Before being used, the bags were tested for small holes or other defects. For this they were immersed in water and air pressure applied to the inside, with the apparatus described by Northrop and Kunitz (6). They were then tested for permeability by being filled with water and subjected to an air pressure from 10 to 20 cm. of Hg. From the time required for small drops of water to appear on the outer surface, their size, and their distribution, a rough idea as to their permeability was obtained.

All osmotic pressure experiments were carried out in a refrigerated chamber at 0°.

[1] The use of Schering's celloidin was suggested to us by a paper of Michaelis and Perlzweig (5).

At the end of each osmotic pressure experiment, the outer solutions were tested for protein to make certain that none had diffused through the membrane. The test used was the appearance of a turbidity on the addition of a tannic acid solution.[2] At the end of an experiment, the inner solution was analyzed for protein. Owing to the presence of urea, the protein content could not be determined by the Kjeldahl method. Colorimetric methods were, therefore, resorted to. For the colorless proteins, a colorimetric method was developed, making use of the modified phenol reagent of Folin and Ciocalteu (7), which reacts with proteins to give a blue color in alkaline solutions. The procedure is similar to that described by Wu (8) for plasma proteins. For a standard, a known quantity of a finely divided preparation of the protein to be analyzed was dissolved in a given volume of urea solution and treated in the same way as the solution to be analyzed. In all cases the water content of the protein preparation was determined and the corresponding correction applied.

Baker's c. p. urea was used in most of the experiments. Kahlbaum's and Baker and Adamson's urea were also employed with no particular differences.

All pH measurements were made with the hydrogen electrode at 25°, with the 0.1 N KCl calomel electrode as the reference cell.

Criteria of Equilibrium.—Of first importance in this work was the assurance that the measurements represented equilibrium values. The criteria accepted by us for equilibrium were (1) that the final value had reached a stationary state and was not changing to higher or lower values; (2) that they could be approached starting from either lower or higher hydrostatic levels than the osmotic pressure; and (3), that the values obtained could be reproduced by duplicate experiments (9). All three of these tests were not carried out in every instance. We assured ourselves that these tests were met in a number of selected cases and then for the most part made use of the criterion of a final unvarying value. A few sample tests illustrating the osmotic pressure equilibrium relations are shown graphically in Fig. 2.[3]

[2] 1 cc. of a freshly prepared 20 per cent solution of tannic acid was added to 5 cc. of the outer solution. Under proper conditions, this test is sensitive to a concentration of protein of 1:100,000.

[3] The increased hydrostatic levels over the osmotic pressure, indicated by the arrows in Fig. 2, which is observed immediately upon removing the

Solvent Properties of 6.66 M Urea Solution.—It was suggested by Spiro (2) that the solvent effect of urea solution for proteins was due to urea acting as a base. The low value for the basic dissocia-

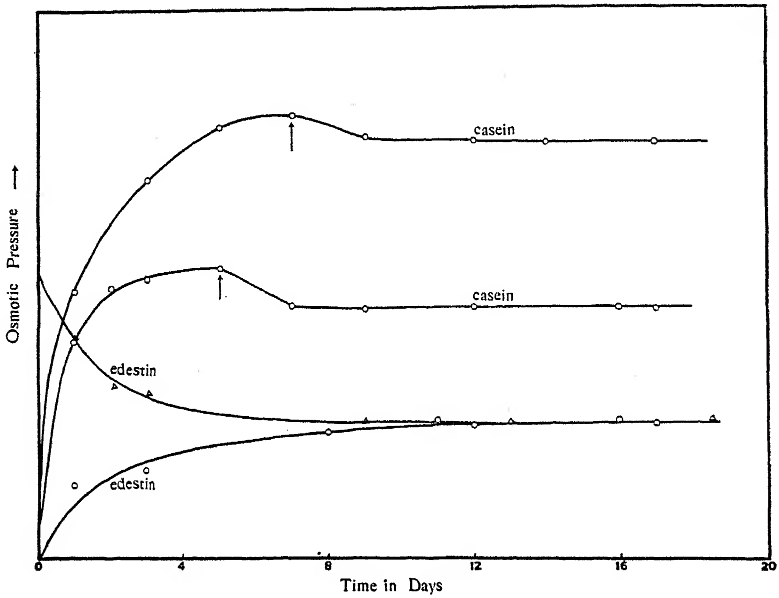

FIG. 2. Rate of attainment and constancy of the osmotic pressure equilibrium. The edestin curves, showing equilibrium is attained starting either from a higher or lower pressure, have been shifted along the ordinate axis so as to coincide, as they represent edestin at different concentrations. The arrows of the casein curves indicate the time at which stirring of the solutions ceased.

tion constant of urea, 1.5×10^{-14} (not much greater than water) is not consistent with the assumption that urea forms salts with

vessels from the rocking apparatus, are to be attributed to (1) strains on the collodion bags produced by their movement through the external solution, and (2) to the tilting in rocking, since the distance up the tube to which the column of liquid rises must be greater when the vessels are in an average inclined position (in rocking) than in the vertical (at rest), in order to exert the same hydrostatic pressure upon the solution. Owing to the slowness of readjustment of the level, the hydrostatic pressure measured in the vertical position immediately after rocking has ceased is greater than the new equilibrium pressure measured after the vessels have been allowed to stand for some time.

acids as weak as proteins. There is, however, a possibility that proteins, which dissociate as very weak acids in aqueous solution, might become stronger, owing to a pure solvent effect, when dissolved in urea solution. Certain analogous instances are known, as shown by the recent work of Hall and Conant (10), in which they demonstrated that certain acids, *e.g.* sulfuric, become even stronger in another solvent (glacial acetic acid). A number of experiments was carried out to test the ionizing properties of urea solutions. To use simpler systems than proteins for these tests, a comparison was made of the hydrogen ion activity[4] and conductivity of equivalent concentrations of acetic acid and potassium dihydrogen phosphate in water and in urea solutions. The results are here tabulated.

	pH	Specific conductivity. *mhos per cc.*
0.05 M HAc in water (11)	2.70	3.45×10^{-4}
0.05 " " " 6.66 M urea	4.01	1.76×10^{-4}
0.05 " KH_2PO_4 in water (11)	4.50	
0.05 " " " 6.66 M urea	5.65	

It is seen that there is a decrease in hydrogen ion activity and a lowered conductivity in the urea solutions. Instead then, of urea acting to increase the strength of acids there is actually produced a lowering of the strength of acids in urea solutions. Solutions of urea, judged on the basis of these experiments, are poorer ionizing solvents than pure water, and salt formation should occur less readily in concentrated urea solution than in water.

In most of our experiments, for reasons to be considered later, buffered urea solutions were employed. Since urea solution is a poorer ionizing solvent than a dilute aqueous solution, the buffer mixtures in common use could not be employed in this work with the expectation that the pH would be the same as found in aqueous solution. The buffer systems employed in all the work were prepared from acetic acid-sodium acetate (CH_3COOH-CH_3COONa) and potassium dihydrogen phosphate-disodium hydrogen phosphate (KH_2PO_4-Na_2HPO_4) mixtures. The titration curves for these buffer systems were determined in 6.66 M urea solution

[4] We have adopted the convention that equal electromotive force readings signify equal pH values in dilute aqueous solution and concentrated urea solutions.

and are shown in Fig. 3. As the curves show, the pH values for given ratios of acid to salt, in the urea solvent, are very considerably different from those in aqueous solution. The comparison curves for the acetate and phosphate buffers in aqueous solution are taken from the data of Sørensen (12) and Walpole (13) as given by Clark (11). From the titration curves it was estimated

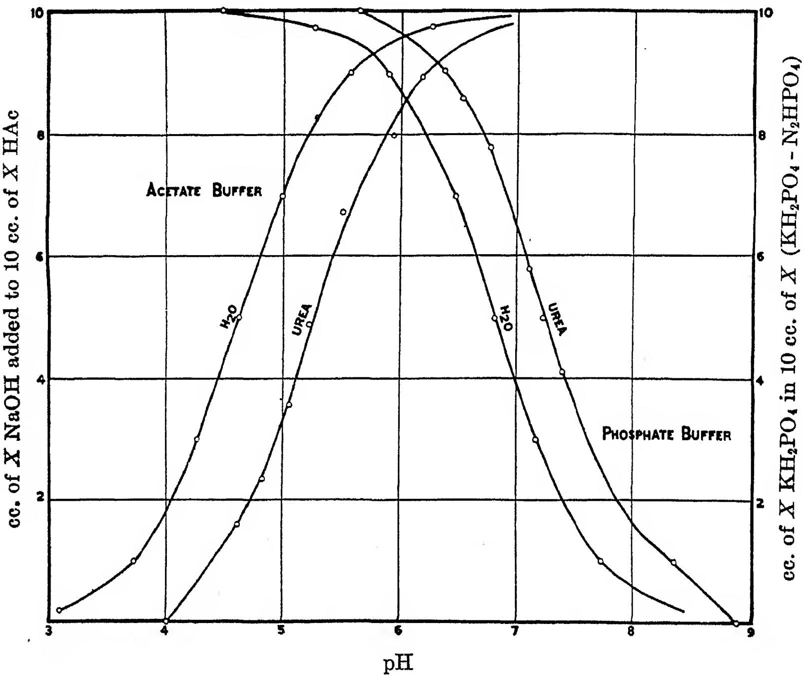

FIG. 3. Titration-dissociation curves of acetate and phosphate buffers in aqueous and 6.66 M urea solvents. For acetate buffer in water, $X = 0.2$ M; in 6.66 M urea, $X = 0.05$ M; phosphate buffer in water, $X = 0.066$ M; in 6.66 M urea, $X = 0.05$ M.

that the apparent strengths of acetic acid and phosphoric acid in 6.66 M urea solution have the values of $pK' = 5.25$ and $pK_2' = 7.22$ respectively.

Casein.

Preliminary Measurements.—All the experiments were carried out on casein prepared from fresh, unpasteurized milk by Van

Slyke and Baker's method (14), with the modifications introduced by Greenberg and Schmidt (15).

The first trial experiments were carried out in two series of measurements with purified casein dissolved in 6.66 M urea solution. The results of the two series with increasing casein concentration are shown in Table I. There is a fair agreement between the results obtained in the two series. These preliminary experiments

TABLE I.

*Osmotic Pressure of Casein in 6.66 M Urea Solution.**

Casein per 100 gm. solvent.	Observed pressure of solution.	Capillary correction.	P	P per unit concentration.
		Series 1.		
gm.	*cm.*	*cm.*	*cm.* H_2O	*cm.* H_2O
0.93	12.5	0.52	13.2	14.2
1.80	26.6	0.43	29.0	16.2
2.48	39.0	0.43	42.7	17.2
3.42	51.0	0.43	56.1	16.4
		Series 2.		
0.87	11.75	0.43	12.4	14.3
3.40	53.2	0.43	58.5	17.2
Osmotic pressure of casein in 6.66 M urea solution + KCl (0.05 M).				
1.05	9.78	0.43	10.3	9.8

* The data of Series 1 and 2 of this table were published in a preliminary paper (16). We were then under the impression that we were measuring the osmotic pressure of isoelectric casein and made a calculation of the molecular weight accordingly. As is shown in this communication, the above conception was erroneous.

demonstrated that the use of a solvent other than water, could be satisfactorily employed for osmotic pressure investigations on proteins. The average value for the osmotic pressure of the casein per unit of concentration amounts to about 16 cm. of water. We had next to be sure whether this value represented the minimum value of the osmotic pressure in this solvent, as would be expected from a solution containing isoelectric casein. To test this, an experiment was carried out in which a neutral salt, potassium

chloride, at a concentration of 0.05 M was added to both the inner and outer solutions. The last line of Table I shows that the osmotic pressure of casein was depressed from 16.0 to 9.8 cm. of water by the addition of the salt. Loeb (17) has shown that the depressing action of a neutral salt (at a low concentration) on the osmotic pressure occurs when the protein is at a hydrogen ion activity other than that of the isoelectric point. When the protein is in solution at the isoelectric point, no depression should occur. From the effect of the potassium chloride in the above experiment, we had to conclude that the casein, even though a pure preparation, was not in solution in the isoelectric state. This seemed to be supported by a determination of the hydrogen ion activity of such a casein solution. There was obtained a pH of 5.35, which is considerably more alkaline than pH 4.62, the isoelectric point for casein in aqueous solution as determined by Michaelis and Pechstein (18). From the previous considerations on the solvent properties of urea solutions, the high osmotic pressure could not be due to salt formation with urea, but was probably due to the base present. Experiments were carried out to determine the sources of this base. The possibility of base being present in the urea was first investigated. No ammonia could be detected by Nessler's test. This test, however, is rendered less sensitive to ammonia by the presence of urea.

The pH of 6.66 M urea solution was found to be more alkaline than the distilled water in which it was prepared. Upon recrystallizing the urea, twice from alcohol and once from water, there was obtained a sample that gave the same hydrogen ion activity as the distilled water in which it was dissolved. Before recrystallization, the Baker's urea had a specific conductivity of 3.80×10^{-5} mhos; after recrystallization, the conductivity was reduced to 1.13×10^{-5} mhos. The urea evidently contains a trace of base. However, this amount of base was shown to be insufficient to account for the obtained osmotic pressures. Measurements were made of the osmotic pressure of casein in solutions of the highly purified urea. The values of osmotic pressure obtained were only 8 per cent lower than in the ordinary Baker C. P. urea. Evidently, the main source of increased alkalinity of casein in urea solution does not arise from the urea.

We next considered the possibility of the basic impurity being

present in the casein itself. The insolubility of casein in water makes it difficult to detect impurities by the conductivity method. The addition of a purified casein to a urea solution prepared from a stock of C. P. sample did not significantly alter the conductivity. However when it was added to a solution prepared from highly purified urea, there was produced a considerable increase in conductivity as shown below.

	Conductivity. *mhos per cc.*
Solution of highly purified urea (6.66 M)............	1.68×10^{-5}
" " " " " (6.66 ") and casein (approximately 0.75 per cent)...................	7.72×10^{-5}
" " (another preparation).....................	1.07×10^{-4}

It seems likely then that the increased osmotic pressure is due to a trace of base present in the dry casein. Evidently the ash in casein prepared by the Van Slyke and Baker method consists of small amounts of base presumably held by the formation of insoluble basic salts of casein. The high osmotic pressure obtained in pure urea solutions and its decrease on the addition of a neutral salt can be explained on the basis of a Donnan membrane equilibrium arising from a small amount of ionized casein. We believe while it would be possible, if proper attention is given to purification of the materials, to obtain reliable osmotic pressure results with casein dissolved in pure urea solutions as solvents, it would be very laborious and for the present impractical.

Osmotic Pressure of Casein in Buffered Urea Solution.

Influence of Hydrogen Ion Activity on the Osmotic Pressure.

In all of the following experiments, buffered solutions of urea were employed to overcome the interfering effects of traces of base present in pure urea solutions containing casein, and to adjust the hydrogen ion activity to the desired pH values. As already stated, acetate and phosphate buffers were employed.

It is essential from the standpoint of molecular weight calculations, to measure the osmotic pressure at the hydrogen ion activity of the isoelectric point. This value, according to the present accepted ideas, represents the hydrogen ion activity at which the osmotic pressure of the protein is a minimum, and where it is free

from the influence of the Donnan membrane equilibrium. Due to the difference in solvent properties, there were good reasons for assuming that the isoelectric point of casein in urea solution would be appreciably different from that in aqueous solution. The effect of the hydrogen ion activity on the osmotic pressure of casein in 6.66 M urea solution was therefore first determined in order to locate the pH at which the osmotic pressure is a minimum. The results of these measurements are plotted in Fig. 4. In Fig. 4, the osmotic pressures per unit of concentration (1 gm. per 100 gm. of solvent) are plotted against pH. It is

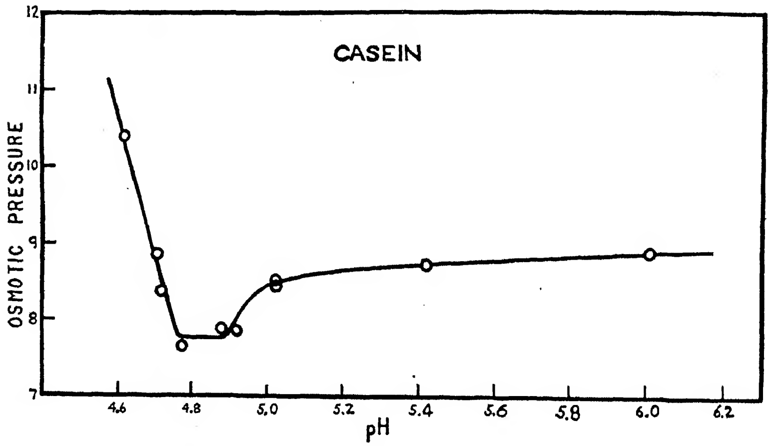

FIG. 4. Influence of the hydrogen ion activity on the osmotic pressure of casein. Location of the hydrogen ion activity at which the osmotic pressure is a minimum, pH 4.78 to 4.92. Ordinates, osmotic pressure per unit of concentration in cm. of water.

seen that the osmotic pressure of casein shows a minimum over the range from about pH 4.78 to pH 4.92. This range is therefore to be considered the isoelectric zone of casein in the 6.66 M urea solution. That the osmotic pressure over this region of pH is due to isoelectric casein is supported by the measurements of the hydrogen ion activity of the inner and outer solutions, which are given in Table II. In the more acid solutions the pH of the inner solution is greater than the outer, showing the casein is positively charged. This difference between inner and outer pH values indicates that there is a considerable membrane potential present.

In the alkaline solutions the reverse is the case, the inner solution is at a more acid pH than the outer, indicating that the casein is negatively charged. In the region between pH 4.78 and 4.92, where the osmotic pressure is at the minimum, the difference in hydrogen ion activity between the outer and inner solutions is practically zero, supporting the supposition that the casein is in solution in the isoelectric condition.

Fig. 4 shows that the influence of hydrogen ion activity on the osmotic pressure of casein is quite different on the acid side of the isoelectric point and on the alkaline side. On the acid side, the osmotic pressure shows marked sensitiveness to changes in pH.

TABLE II.

Osmotic Pressure and pH Difference of Casein.

Casein per 100 gm. solvent.	*P* per unit concentration.	pH, inner solution.	pH, outer solution.	ΔpH
gm.	*cm.* H_2O			
0.94	10.40	4.62	4.55	+0.07
1.06	8.85	4.71	4.64	+0.07
1.03	8.50	4.72	4.66	+0.06
0.88	7.66	4.78	4.76	+0.02
1.09	7.87	4.88	4.87	+0.01
1.05	7.84	4.91	4.90	+0.01
0.91	8.43	5.02	5.04	−0.02
1.03	8.50	5.04	5.05	−0.01
0.93	8.70	5.42	5.45	−0.03
1.05	8.88	6.09	6.17	−0.08

On the alkaline side, the pressure, except for a slight rise close to the isoelectric region, is but little influenced by the hydrogen ion activity. These general characteristics of the marked increases of osmotic pressure on the acid side of the isoelectric region and only slight changes on the alkaline side were found with all the proteins studied. For the present, we are only able to offer a qualitative explanation of this phenomenon. The factors that would be expected to govern the changes in osmotic pressure on either side of the isoelectric point are: (1) the extent of the ionization of the protein at different pH values, and (2) the depressing action of the buffer salts. The ionization of the protein, in this case casein in urea solution, is unknown, so its effect on the os-

motic pressure-pH curve cannot be estimated. In aqueous solution, casein is known to have a slightly sharper increase in ionization on the acid side of the isoelectric point than on the alkaline side up to about pH 6.0 (19). If the relations are similar in urea solution, the titration curve of casein and the influence of the buffer salts offer a qualitative explanation for the casein curve. The depressing action of a salt has been shown by Loeb (17) to be almost solely due to the ion of opposite charge to that carried by the protein and to be approximately proportional to the concentration of that ion. With casein, where acetate was used for buffering (0.05 M with respect to total acetate), sodium ion is responsible for the depression on the alkaline side and acetate ion on the acid side of the isoelectric point. In the acid region, the buffer is mostly present as undissociated acetic acid which would give but a low concentration of acetate ions to produce a diminution of the osmotic pressure, while in the alkaline region the buffer is largely present as sodium acetate which yields a considerable concentration of sodium ion acting to decrease the osmotic pressure.

Relationship between Osmotic Pressure and Concentration of Casein.

After determining the isoelectric region of casein, two series of osmotic pressure measurements were made with varying concentrations of casein in 6.66 M urea solution at the pH of the isoelectric point. The results obtained are shown plotted in Curve 1 of Fig. 5. In Fig. 5, the concentration of casein is expressed in gm. of dry weight of casein per 100 gm. of solvent. The plot obtained shows that the osmotic pressure is not proportional to increasing concentration of casein, but instead is concave to the concentration axis, giving greater than proportional increases of osmotic pressure. This same type of curve, as will be seen later, was obtained with the other proteins studied by us. In aqueous solutions of pure proteins, this upward trend with increasing protein concentration has been observed by Adair (9, 20, 21) for hemoglobin, and Kunitz (22) for gelatin. Nor is this phenomenon restricted to proteins or other colloids. A similar curvature has been noted at high concentrations for the osmotic pressure of sugar solutions (23). The underlying cause for this phenomenon is evidently a very general thing operating for all proteins in widely divergent types of solutions. Indeed the only difference between proteins and such crys-

talloids as sugars, in this respect, is that the divergence from proportionality with the former is noted in solutions of far lower concentration.

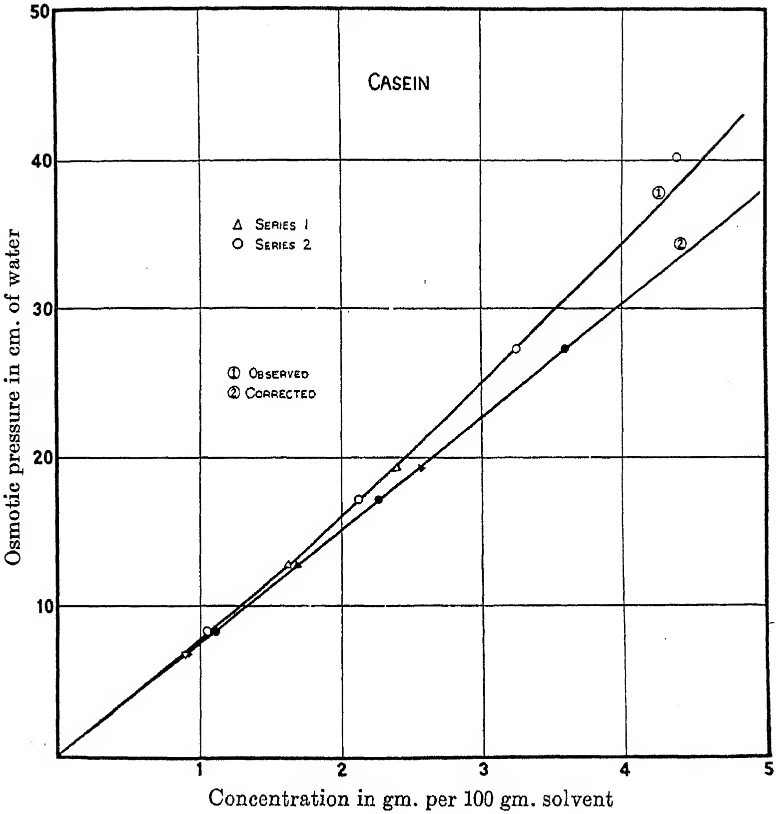

FIG. 5. Relationship between the concentration and the osmotic pressure of casein at the isoelectric point (pH 4.78 to 4.92). Curve 1 becomes linear (Curve 2) by correction of the concentration for solvation.

For gelatin, Kunitz (22) has proposed that the deviations of the osmotic pressure from proportionality to the concentration are due to a hydration of this protein. On the other hand, Adair (21), from his studies of the osmotic pressure of hemoglobin, ascribes such deviations to a failure of the hemoglobin solutions to obey the law of van't Hoff. That solvation does occur in protein systems is

fairly certain. Density measurements on proteins by Chick and Martin (24) and Svedberg (25), viscosity studies by Chick and Lubrzynska (26), du Noüy (27), and Kunitz (28) leave little doubt as to the occurrence of this phenomenon. We are of the opinion that solvation is a factor in accounting for the deviations of isoelectric protein solutions from the laws of perfect solutions as exemplified by the curvature of the osmotic pressure-concentration curve. However, we by no means maintain that solvation is the only factor causing deviations from the law of ideal solutions in protein systems. In all likelihood, the departure of the osmotic pressure from proportionality to concentration is a result of more than one cause. The question confronting us is how to distinguish between the various causes involved and at what concentrations do they become of significance? This question is of course for the present unanswerable.

Over the range of concentration employed by us, the relation between osmotic pressure and concentration fits the equation:

$$P = K \frac{100\,C'}{100 - hC'} \qquad (1)^{5}$$

in which P is the osmotic pressure, C' the measured concentration, and K and h are constants. If the deviation of the osmotic pressure is due to protein solvation and it is further assumed that the amount of solvation per gm. of protein is constant, then Equation 1 can be quite rationally derived. It is quite evident that if solvation takes place, the concentration of protein as analyzed, or as initially made up and expressed, either as gm. per 100 cc. of solution or as in this investigation, in gm. per 100 gm. of solvent, does not represent the true (or thermodynamic) concentration of the protein in solution. It is also obvious that the thermodynamic concentration is the quantity that regulates the magnitude of the osmotic pressure and is therefore the one that should be used. The osmotic pressure should then be related to the thermodynamic concentration in a perfect solution so that:

$$P = KC \qquad (2)$$

[5] The constant K in this equation is not equivalent to the usual term RT because of the concentration unit employed, but is proportional to RT.

in which K is a constant and C is the thermodynamic concentration. If h is considered the number of gm. of solvent of solvation (gm. of solvent held per 1 gm. of protein) then $100 - hC'$ represents the weight of free solvent. The thermodynamic concentration, expressed in gm. per 100 gm. of solvent, becomes:

$$C = \frac{100\,C'}{100 - hC'} \tag{3}$$

and from Equations 2 and 3 there is obtained Equation 1 as already given.

TABLE III.

Valuation of P : C of Casein.

P	C', per 100 gm. solvent.	$P:C'$	h	C	$P:C$
cm. H_2O	*gm.*				
6.74	0.88	7.66	2.80	0.90	7.48
8.24	1.05	7.84	2.80	1.08	7.62
12.71	1.62	7.82	2.80	1.70	7.48
17.10	2.12	8.07	2.80	2.25	7.59
19.31	2.38	8.12	2.80	2.55	7.57
27.21	3.25	8.37	2.80	3.58	7.60
40.20	4.37	9.18	2.80	4.98	8.05*
Mean value..					7.54

* This value was not used in obtaining the mean.

Also from Equation 1, if V be the reciprocal of C' and h' be $\frac{h}{100}$, there is readily obtained:

$$P\,(V - h') = K \tag{4}$$

which is the form of equation used by Sackur (29) and Porter (30) to fit the osmotic pressure-concentration data of sugar solutions and other crystalloids. This equation is of course also analogous to the Van der Waal equation of the state of gases, in which the constant h' is taken to represent the actual volume of the solute. Since the value of h' is greater than can be accounted for by the volume of the sugar in the solution, Porter assumes that its magnitude is due to hydration of the sugar molecules. Here too it is to

be seen that there is a close relationship between the osmotic pressure of crystalloids and proteins and that the differences in degree are due to the huge size of the protein molecule.

It is to be seen that the term h is a function regulating the deviation of the osmotic pressure-concentration curve from proportion-

TABLE IV.

Ratios of Osmotic Pressure to Concentration of Hemoglobin (0°). Corrected by Equation 1. Data of Adair ((21) Table X).

C, Hb per 100 cc. solution.	Density* of protein solution.	C', Hb per 100 gm. solvent.	C,† corrected by Equation 1.	Pp	$Pp : C$
gm.		*gm.*		*mm. Hg*	
0.68	1.0142	0.67	0.67	1.9	2.81
2.21	1.0179	2.20	2.28	6.2	2.79
2.90	1.0196	2.93	3.07	8.5	2.76
3.58	1.0212	3.64	3.86	11.1	2.87
5.00	1.0246	5.12	5.53	14.6	2.64
8.00	1.0319	8.42	9.75	27.7	2.84
8.12	1.0322	8.53	9.90	28.2	2.85
10.00	1.0367	10.69	12.93	38.2	2.95
12.00	1.0415	13.04	16.53	47.5	2.87
15.50	1.0499	17.34	24.09	64.6	2.68
19.40	1.0593	22.44	35.25	99.2	2.81
19.80	1.0603	23.00	36.89	100.4	2.72
20.00	1.0608	23.22	37.22	105.2	2.82
24.00	1.0704	28.90	54.34	146.5	2.70
25.00	1.0723	30.42	59.97	169.5	2.82
28.00	1.0801	35.18	81.79	227.8	2.78
29.00	1.0825	36.68	89.67	248.1	2.76
34.40	1.0955	45.80	177.50	352.2	1.98

* We are indebted to G. S. Adair for privately supplying us the density values required in the calculations of this table; also for reading the manuscript of this communication and making certain valuable suggestions.

† Calculated from $C = \dfrac{100\ C'}{100 - 1.62\ C'}$.

ality. Its value will be such that K, the osmotic pressure per unit of concentration, will be a constant. To determine C, the thermodynamic concentration of the protein, h must be so chosen that this condition is fulfilled. This treatment of the data on the osmotic pressure of casein at the isoelectric point is given in Table III. The values for C given in the fifth column are obtained from

C' (second column) by Equation 3, h being considered equal to 2.80. The last column shows the constancy of the osmotic pressure per unit of concentration when the values of C are employed.

Adair (21) has employed Equation 1 to represent the relationship between osmotic pressure and concentration of hemoglobin up to about 5 per cent. At higher concentrations than this, Adair states that this simple equation no longer holds. It should be

TABLE V.

Ratios of Osmotic Pressure to Concentration of Gelatin (35°).
Corrected according to the method of Kunitz and by Equation 1.

P	C', per 100 cc. H_2O.	C,* per 100 cc. H_2O.	H_2O of hydration (h in Equation 1).	H_2O of hydration (Kunitz).	$P:C$ (Kunitz).	$P:C$ by Equation 1.
mm. Hg	*gm.*	*gm.*	*cc. per gm.*	*cc. per gm.*		
3.5	1.01	1.06	4.68	7.00	3.24	3.31
7.5	2.03	2.24	4.68	6.78	3.19	3.34
12.0	3.07	3.56	4.68	6.52	3.12	3.37
17.0	4.12	5.12	4.68	6.30	3.06	3.32
23.0	5.19	6.86	4.68	5.93	3.07	3.35
29.5	6.28	8.89	4.68	5.60	3.04	3.32
47.0	8.52	14.17	4.68	4.95	3.20	3.32
52.0	9.00	15.56	4.68	4.65†	3.21	3.34
63.0	10.00	18.82	4.68	4.32	3.46	3.34
76.5	11.00	22.68	4.68	4.05	3.70	3.37
92.0	12.00	27.41	4.68	3.78	3.95	3.35
112.0	13.00	33.30	4.68	3.56	4.34	3.33
135.0	14.00	40.60	4.68	3.35	4.72	3.32

* Calculated from $C = \dfrac{100\ C'}{100 - 4.68\ C'}$.

† The values from here on were obtained by the use of Kunitz's Equation 6 (22).

noted that Adair expresses the concentration of the hemoglobin solutions used by him in terms of gm. of protein per 100 cc. of solution. If his results are recalculated to gm. of protein per 100 gm. of solvent a good agreement of his data is obtained up to nearly 30 per cent hemoglobin concentration employing a value of 1.62 for h. This is shown in Table IV. In his measurements on gelatin, Kunitz (22), while employing the hypothesis of hydration to account for the results, arrives at values of diminishing hydration with increasing gelatin concentration. It can readily be shown that a better agreement of the data of Kunitz can be ob-

tained by Equation 1, with a value for h of 4.68, than by the method employed by him. This is illustrated in Table V. If the deviation of the osmotic pressure of isoelectric proteins with increasing protein concentration is due to a single cause, namely solvation, then the values of h obtained, 2.80 gm. of solvent per gm. of casein, hemoglobin, and edestin in 6.66 M urea solution, 1.62 gm. of H_2O per gm. of hemoglobin, and 4.68 gm. of H_2O per gm. of

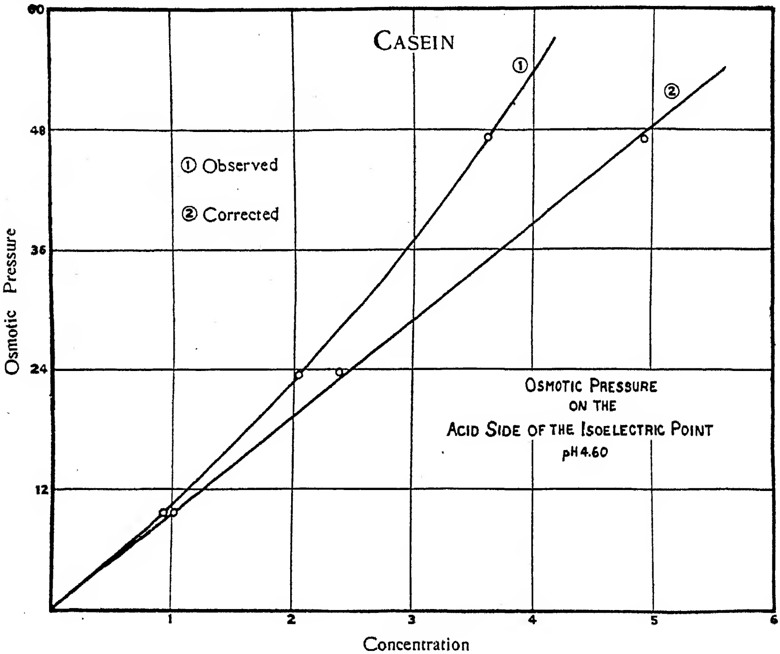

FIG. 6. Relationship between the osmotic pressure and concentration of casein on the acid side of the isoelectric point. Ordinates, pressure in cm. of water.

gelatin may be considered as fairly accurate estimates of the degree of solvation of these proteins respectively. On the other hand, if the solvation hypothesis is in error, then the factor h is merely an empirical constant.

Relationship of Osmotic Pressure to Concentration of Casein in Non-Isoelectric Solution.

A few experiments were carried out on the osmotic pressure of casein with increasing protein concentration, quite similarly to

those previously described, except that the inner solutions of casein were initially adjusted to a pH of 4.60, a hydrogen ion activity on the acid side of the isoelectric point of casein in 6.66 M urea solution. The results are shown graphically in Fig. 6. A glance at this curve shows the deviation of osmotic pressure from proportionality to concentration to be much greater than observed for isoelectric solutions of casein (Fig. 5). Due to the acquired charge of the casein molecule, it is quite plausible that there should be an increase in the degree of solvation. While it may be shown (Table VI) that the value of 7.37 for h corrects the concentration, so that the osmotic pressure conforms to Equation 2, such a value of h cannot have the same significance it does under isoelectric conditions. In non-isoelectric solutions a Donnan

TABLE VI.

Membrane Potential-Protein Concentration Relationship of Ionized Casein.

C', per 100 gm. solvent.	C*	$P:C'$	$P:C$	pH, inner solution.	pH, outer solution.	ΔpH
gm.						
0.94	1.01	10.40	9.67	4.62	4.55	0.07
2.04	2.40	11.52	9.78	4.68	4.59	0.09
3.61	4.93	13.08	9.60	4.79	4.60	0.19

* Calculated from $C = \frac{100\ C'}{100 - 7.37\ C'}$.

membrane equilibrium is present, and the pressure resulting therefrom, also increases to more than is proportional to increases in protein concentration. This has been shown from theoretical considerations by Marrack and Hewitt (31); and our experiments (Table VI) show an increasing membrane potential with increasing protein concentration. Because the effects produced by charge and by solvation influence the osmotic pressure-concentration curve in the same manner, the value of h must express the sum of these two effects at hydrogen ion activities other than the isoelectric point.

Molecular Weight of Casein.

The use of the van't Hoff osmotic pressure-volume-concentration equation to calculate the molecular weights of proteins as is

customarily done, is open to objection (32, 33). The thermodynamic equation for the osmotic pressure of a substance, based upon weight concentration, is now generally agreed to be the more exact one. A general form of such an equation is given by Lewis and Randall (34) and is:

$$P = N_1 \frac{RT}{Vo} \tag{5}$$

in which N_1 is the mol fraction of the solute, Vo the molal volume of the solvent,[6] and R and T have their usual significance.

This equation, it can be shown, is applicable to the calculation of the molecular weight of a substance in any solvent or any mixed solvent of any number of components from the following considerations. The mol fraction

$$N_1 = \frac{n_1}{n_1 + n_2 + n_3 \ldots} \tag{6}$$

in which n_1 is the number of mols of solute in 100 gm. of solvent and n_2, n_3, . . . are the number of mols of each component of the solvent per 100 gm. of solvent.

Let $n = n_2, n_3$ . . . for all of the components permeable to the collodion membrane, then

$$P = \frac{n_1}{n_1 + n} \cdot \frac{RT}{Vo} = \frac{n_1 RT}{n_1 Vo + nVo} \tag{7}$$

For substances of high molecular weight, n_1 is extremely small compared to n and hence $n_1 Vo$ may be neglected. The osmotic pressure equation then becomes

$$P = \frac{n_1 RT}{nVo} \tag{8}$$

Further,

$$nVo = V = \frac{W}{d} \tag{9}$$

where V is the volume of the solvent in which n_1 mols of solute are dissolved, W is the weight of the solvent in which n_1 mols of solute are dissolved, and d is the density of the solvent.

[6] For a mixed solvent, the volume of solvent that contains Avogadro's number of molecules, irrespective of their kind, can be taken as the molal volume (see (35) p. 315).

For the special case where concentration is expressed in terms of gm. of solute per 100 gm. of solvent,

$$\frac{W}{d} = \frac{100}{d} \tag{10}$$

Then obviously,

$$P = \frac{n_1 dRT}{100} \tag{11}$$

If now there is placed, $n_1 = \frac{C}{M}$ in which C is the number of gm. of solute dissolved in 100 gm. of solvent and M is the molecular weight of the solute, there is obtained by substitution,

$$P = \frac{CdRT}{100M} \tag{12}$$

and

$$M = \frac{CdRT}{100P}. \tag{13}$$

The temperature at which the osmotic pressure experiments were carried out was 0°. The density of a 6.66 M urea solution at this temperature is 1.097. We accordingly evaluate the constants of Equations 11 and 12 as follows:

$$R = \frac{22.414 \times 1000 \times 760 \times 13.597}{10} = 84{,}800 \text{ cc.-cm. of } H_2O \text{ per degree}$$

We then put $M = \frac{C}{P} \times \frac{1.097 \times 84{,}800 \times 273.1}{100}$ which reduces to $M = \frac{C}{P} \times 2.54 \times 10^5$, when the osmotic pressure is measured in cm. height of a column of density 1, and concentration of solute in gm. per 100 gm. of 6.66 M urea solution. In other words, 2.54×10^5 is the osmotic pressure in cm. of water ($d = 1$) that 1 mol of a substance dissolved in 100 gm. of 6.66 M urea solution at 0° would exert.

Similarly, the value 2.315×10^5 is the osmotic pressure in cm. of water that 1 mol of a substance dissolved in 100 gm. of water

at 0° would exert. From Table III, the average value of $P:C$ for casein is 7.54. When this value is introduced into Equation 13 there is obtained,

$$M = \frac{1}{7.54} \times 2.54 \times 10^5 = 33{,}600 \pm 250$$

as the molecular weight of casein.

The osmotic pressure method of determining molecular weight has the shortcoming that it does not permit us to decide whether we are dealing with a single molecular species or a mixture. The value of 33,600 obtained for the molecular weight of casein may thus be only the average value for the components present. There is evidence from the work of Linderstrøm-Lang (36) that casein is not a homogeneous substance but is a mixture of proteins. Even so, the results obtained by us have the significant value of being the average molecular weight of the casein component of cow's milk provided, of course, that no change in the state of aggregation has been produced by the urea.

Hemoglobin.

Preparation and Purification.—The method used for preparing hemoglobin is based upon the electrodialytic procedure of Stadie and Ross (37). The aim of the preparation was to obtain a small amount of hemoglobin in a high state of purity in a comparatively short time, so that freshly prepared hemoglobin might be used for each set of experiments. The procedure employed is as follows: The cells from freshly drawn horse blood were washed three or four times with an equal volume of 1.75 per cent NaCl solution; separation from the saline was made by centrifuging for 15 minute periods. The washed mass of cells was hemolyzed by adding an equal volume of toluene, shaken in a mechanical shaker for an hour or so, and allowed to stand overnight. Upon centrifuging this mixture, a separation into three layers is obtained: the lowest, a precipitate of crystallized hemoglobin (and unhemolyzed cells, if hemolysis is not complete), a middle layer of concentrated solution of hemoglobin, and an upper layer of a mixture of toluene and swollen stroma. These layers are separated and the middle concentrated hemoglobin solution is poured into the membrane

compartment of an electrodialysis box (37) and the hemoglobin is caused to crystallize out by removal of the base holding it in solution.

These crystals are further purified by repeating the electrodialysis, 0.2 M Na_2HPO_4 being used to bring the precipitated hemoglobin into solution again. If, during the preparation up to this stage, some denatured hemoglobin is formed, it is separated from normal hemoglobin at this point, as disodium phosphate solution is not sufficiently alkaline to dissolve denatured hemoglobin.

The oxyhemoglobin obtained by crystallization from basic phosphate solution shows an appreciable conductivity. It is therefore probable that crystallization of the hemoglobin from a concentrated solution occurs not when all the base with which it is combined, is removed, but when it is reduced to a very small quantity. Hence, it is not possible to obtain hemoglobin entirely free from electrolytes by repetition of this procedure.

The removal of the final traces of base present in the crystals was accomplished by electrodialysis of a dilute solution of hemoglobin. We found it advantageous to carry out this electrodialysis in collodion bags of about 50 cc. capacity, placed between plate carbon electrodes set in a large beaker, to which were applied a 110 volt direct current. Distilled water was kept in constant flow. The extent to which the hemoglobin was freed from electrolytes was followed by measurements on the conductivity of the solution. After 3 to 5 hours of dialyzing a constant conductivity was reached. The purest hemoglobin solution obtained by this procedure had a conductivity of 9.8×10^{-6} mhos at 20°.

Osmotic Pressure of Hemoglobin in Aqueous Solution.—Hemoglobin offered us the opportunity to check our measurements against a protein the molecular weight of which has been determined. To assure ourselves that our preparations gave pure unchanged hemoglobin, we made a few trial measurements in aqueous solution.

The first osmotic pressure experiments were carried out on hemoglobin in pure water. The water used was either boiled, distilled, or double distilled. If care is taken to prevent the reabsorption of CO_2, such water will have a pH of about the isoelectric point of hemoglobin, 6.7. The purity of the hemoglobin

with respect to electrolytes was determined by conductivity measurements on solutions of this protein. Preliminary experiments showed that if the hemoglobin was not freshly prepared, and of high purity, high values of the osmotic pressure were always obtained. For example, hemoglobin solutions whose conductivity ranged from 3×10^{-5} to 1.5×10^{-5} mhos, gave values for the osmotic pressure ranging from about 6.0 to 4.0 cm. of water per unit of concentration. The results given in Table VII were obtained on hemoglobin which was crystallized three times by the procedure already described. The final product was then electrodialyzed to remove the last traces of electrolytes. The dialysis was carried out in the inside of a large desiccator to prevent absorption of CO_2, the current wires and water tubes passing through a

TABLE VII.

Osmotic Pressure of Hemoglobin in Aqueous Solution.

Solution.	P	C'	pH	C*	P_{Na}	P_{Hb}	$P:C$	M
Hb in H_2O†	20.22	5.07		5.52		20.22	3.66	63,200
" " " †	20.22	5.07		5.52	2.94	17.28	3.13	73,900
" " buffer solution	6.25	1.86	6.72	1.91		6.25	3.26	71,000

* Concentration corrected for solvation.

† The specific conductivity of this solution, corrected for the conductivity of the solvent, was 1.38×10^{-5} mhos.

rubber stopper in the desiccator top. Distilled water (conductivity = 4×10^{-6}) was circulated through the system at a rate of 300 cc. per minute for 4 hours. The entire preparation and purification required 4 days.

The osmotic pressure of hemoglobin in the presence of 0.0275 M phosphate buffer at the pH of the isoelectric point, 6.70, was also measured.

In the present experiments, the hemoglobin was analyzed by the excellent colorimetric method of Stadie (38). The hemoglobin in the standard solution was determined from the weight of the residue after evaporation and drying a purified sample to constant weight at 105°. The amount of ash present in the sample was negligible. The results obtained are given in Table VII. The value obtained for the molecular weight in the buffered solution is

71,000. For the measurement in pure distilled water, when no correction is applied for the residual base present, there is obtained a value of 63,400. However the conductivity of the hemoglobin-containing solution at the end of the experiment was 1.38×10^{-5} (18°). We have been able to purify a hemoglobin solution to a conductivity of 9.8×10^{-6}. This indicates that the hemoglobin of this experiment still contained a slight amount of combined sodium, probably from the method of preparation. If all of the conductivity is due to ionized sodium hemoglobinate, a rough calculation of the amount of Na combined with the hemoglobin and effective in increasing the osmotic pressure, can be made. Assuming sodium hemoglobinate to have about the same equivalent conductivity as NaCl, and applying the Kohlrausch equation (39), there are calculated to be 1.27×10^{-4} equivalents per liter of solution of Na present. This concentration of Na will exert an osmotic pressure of $0.127 \times 10^{-4} \times 2.315 \times 10^{5*} = 2.94$ cm. of H_2O. From the osmotic pressure of hemoglobin corrected for the amount of pressure due to the sodium there is obtained a value of the molecular weight of 74,100. In making the calculations the measured concentration of hemoglobin was corrected to the thermodynamic concentration (Equation 1) by using the value of $h = 1.62$ obtained from the data of Adair (20). The correction applied for the amount of residual base present undoubtedly gives too high a value of base in combination with the hemoglobin. The results obtained by us, in aqueous solution, are in fair agreement with those of Adair (20, 21) and Svedberg and Nichols (40) and give assurance that the hemoglobin used in these and the following experiments was normal unmodified hemoglobin.

Osmotic Pressure of Hemoglobin in Urea Solutions.

Effect of pH on the Osmotic Pressure.—As with casein, since the isoelectric point of hemoglobin in urea solution may be appreciably different from that in aqueous solution, the influence of hydrogen ion activity upon the osmotic pressure was first investigated to determine the pH at which the osmotic pressure is at a minimum.

The outer solutions were 6.66 M urea solutions, adjusted to various pH values with phosphate buffers, the phosphate concen-

* See 219 for the significance of this factor.

tration solution being maintained at 0.05 molar. The inner solutions were prepared by dissolving twice crystallized hemoglobin in the outer solutions.[7]

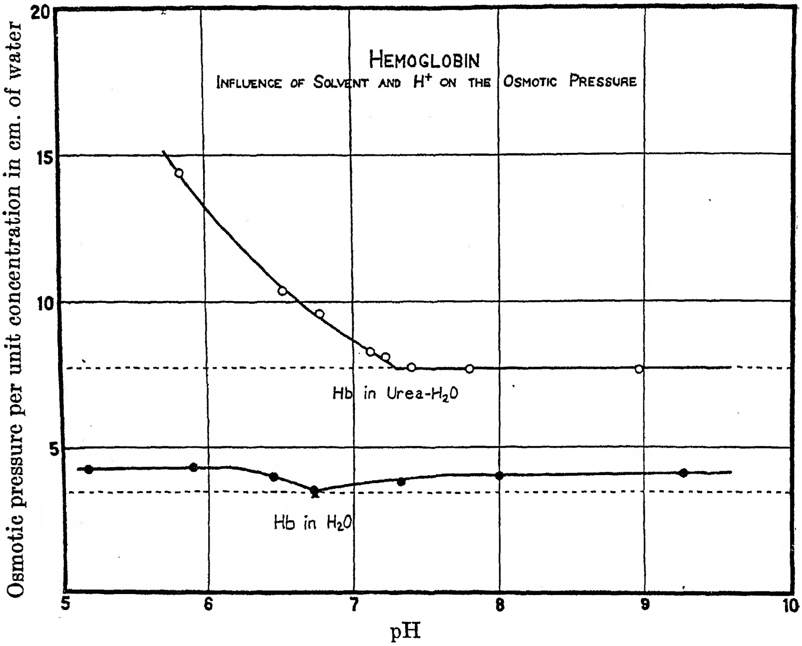

FIG. 7. The osmotic pressure of hemoglobin in different solvents. Dotted lines represent the minimum osmotic pressure in each solvent, corresponding to that of hemoglobin without the presence of a Donnan membrane equilibrium.

The results obtained are shown graphically in Fig. 7 (upper curve). A glance at the curve shows that hemoglobin exhibits a

[7] In the experiments with hemoglobin in urea solution, a number of colorimetric methods were employed in analyzing for hemoglobin. They were: (1) a direct comparison of the natural color of a given solution suitably diluted in the colorimeter against that of a standard hemoglobin solution prepared in the same way and at the same time as the unknown solution; (2) direct comparison as above with the addition of 0.2 M NaOH to both standard and unknown (this changes the color of hemoglobin in urea from orange to yellow); (3) by the acid hematin method (41); and (4) by the method used for analysis of the colorless proteins with the phenol reagent. In dilute solutions of hemoglobin the natural color causes but little interference

constant osmotic pressure over a fairly wide range of hydrogen ion activity. This range occurs in alkaline solutions from pH 7.3 to over 9.0, the most alkaline point measured. The lower curve shows the change of osmotic pressure with pH in aqueous solution buffered with 0.066 M phosphate, as taken from the results of Adair (21). The curves show that the minimum osmotic pressure in aqueous solution is about half the value obtained in urea solution and that a very greatly altered shape of curve is obtained in urea solution.

TABLE VIII.

Relation of the Osmotic Pressure to the Concentration of Hemoglobin.

P	C', Hb per 100 gm. solvent.	$P:C'$	h	C	$P:C$
cm. H_2O	*gm.*				
11.83	1.53	7.72	2.80	1.60	7.41
12.48	1.60	7.80	2.80	1.67	7.44
12.85	1.67	7.68	2.80	1.75	7.33
13.57	1.78	7.62	2.80	1.87	7.26
26.04	3.22	8.14	2.80	3.54	7.35
27.81	3.31	8.40	2.80	3.64	7.65
47.80	5.01	9.54	2.80	5.83	8.23*
Mean value..					7.40

* This value was not used in obtaining the mean.

Variation of the Osmotic Pressure of Hemoglobin with Concentration.

The osmotic pressures of increasing concentrations of hemoglobin in 6.66 M urea solutions were measured at the pH of the minimum osmotic pressure, as previously determined. The relationship between the osmotic pressure and concentration of hemoglobin is similar to that of casein, increasing more than is proportional to the measured concentration. As with casein, a fairly constant value of the expression $P:C$ is obtained if the thermodynamic

with this method. The acid hematin method was found to be the most satisfactory for analyzing solutions of different pH values. The standard solution of hemoglobin was made up in the same solvent as the unknown (6.66 M urea, 0.05 M phosphate buffer) and was prepared from a pure aqueous solution of hemoglobin, the protein content of which was determined by evaporation and drying to constant weight at 105°.

concentration of hemoglobin in solution is calculated by correcting the analyzed concentration by a constant value for the term h. This treatment of the data on the hemoglobin concentration series is given in Table VIII.

It is to be noted that the same value for h, namely 2.80, is required to correct for hemoglobin as for casein. From Table VIII the average value for the osmotic pressure per unit of concentration is 7.40 cm. of H_2O. The molecular weight of hemoglobin in the urea solvent is, therefore, by Equation 12,

$$M = \frac{1}{7.40} \times 2.54 \times 10^5 = 34{,}300 \pm 425$$

DISCUSSION.

The measurements of the osmotic pressure in aqueous solution showed that the hemoglobin we used had the normal molecular weight of 68,000. When this hemoglobin was dissolved in a solution of urea (6.66 M), there was obtained a molecular weight of 34,300. The osmotic pressure-pH curve obtained in urea solution varies most radically from that found in aqueous solution. From what is known of the power of natural hemoglobin to combine with acid and base, this curve cannot qualitatively be explained on the basis of a Donnan membrane equilibrium resulting from the ionization of hemoglobin and the depressing action of the ions from the buffer present. The combination of acid and alkali with hemoglobin, as shown by the investigations of Lewis (42), Felix and Buchner (43), and Hastings and his associates (44) increases rapidly and to about an equal extent (for a short pH range) on both sides of the isoelectric point. The osmotic pressure should be expected to parallel this combination, especially as the depressing action of the phosphate buffer is about constant over the entire pH range. This is illustrated by Adair's curve in aqueous solution shown in Fig. 7.

The increasing osmotic pressure of hemoglobin in acid solutions of urea, the constancy in alkaline solutions, and the double values of the osmotic pressure per unit of concentration obtained in the isoelectric region, are, therefore, not in accord with the electrochemical properties of this protein in its normal state in aqueous solutions. It can be readily shown that an apparent irreversible

change has been produced in the hemoglobin dissolved in urea solution. If the solutions of urea containing hemoglobin at a pH of the isoelectric region were diluted with water, after equilibrium in the osmometers had been reached, immediate and complete precipitation of the hemoglobin occurred. The hemoglobin thus precipitated resembles the insoluble product which results when an aqueous solution of hemoglobin is coagulated by heat. Urea therefore acts upon hemoglobin causing it to become denatured. This action of urea has also been observed by Anson and Mirsky (45). On the addition of urea to a solution of hemoglobin in water, the normal hemoglobin bands (centers at 579 and 543 $\mu\mu$ respectively) gradually fade out, especially if the solution is warmed. If such a solution of hemoglobin containing urea has stood for some time, there can be distinguished a broad, faint, and rather diffuse band in the green part of the spectrum (center at 522 to 525 $\mu\mu$) and a still fainter, narrow band in the neighborhood of 565 $\mu\mu$. A hemoglobin with bands of this character has been studied by Van Klaveren (46) and Keilin (47) and has been named cathemoglobin. It would seem to be identical with denatured methemoglobin. The finding that hemoglobin, denatured by being dissolved in concentrated urea solution, has a molecular weight half that of the original hemoglobin is of great significance. To our knowledge this is the first proof that in denaturation there may occur an actual splitting of the protein molecule. Because of the mildness of urea as a chemical reagent there can be no question of a hydrolysis taking place of the same type as caused by the action of acids, alkalies, or enzymes.

The denaturation of the hemoglobin offers an explanation as to the cause of the constancy of the osmotic pressure of hemoglobin in 6.66 M urea solution, in the alkaline range. According to the work of Adolf (48) on serum globulin, and Hendrix and Wilson (49) on egg albumin, denaturation of a protein causes a decrease in the base-binding power in the pH region of the isoelectric point. Denatured hemoglobin precipitated out from a solution of urea, likewise, does not become appreciably ionized on the addition of base in the presence of salts in solution as alkaline as pH 9.25. This is shown by its insolubility in aqueous buffer solution up to this pH value. Because denatured hemoglobin does not ionize in weakly alkaline solutions, it cannot give rise to a

Donnan osmotic pressure, and hence, as our experiments show, the osmotic pressure does not increase with increasing alkalinity. On the other hand, the hemoglobin in 6.66 M urea solutions at greater acidities than pH 7.3 does not coagulate when the content of urea is decreased. Here the hemoglobin is combined with acid. If a few drops of dilute alkali are added, sufficient to neutralize the combined acid, there is produced an immediate and complete precipitation of the hemoglobin. Hence, in the acid region the osmotic pressure increases with increasing acidity.

Molecular Weight of Hemoglobin in Glycerol.

Finding a molecular weight for hemoglobin in urea solution of half the value in aqueous solution, we were tempted to try another solvent in order to determine whether the effect was due to a change of solvent or to denaturation of the hemoglobin itself. A measurement was therefore carried out on the osmotic pressure of hemoglobin with 6.50 M glycerol as a solvent (65 per cent glycerol solution). This corresponds to nearly the same molar concentration as the urea solutions used. The glycerol was buffered with 0.0275 M phosphate adjusted to the isoelectric point of hemoglobin in aqueous solution.

No measurements were made on the influence of the glycerol solvent on the pH value of the buffer or effect upon the isoelectric point of hemoglobin. However, there are a number of reasons for believing that this adjustment gave a pH very close to the isoelectric point of the hemoglobin. Glycerol shows no ionization, is liquid, and non-nitrogenous, resembling water in these properties as a solvent, and hence would be expected to produce little change in the dissociation of the buffer. Furthermore, as it was found that the shift in the pH of the buffer solutions and the isoelectric regions of the proteins in urea solution were in the same direction, it is quite likely that the pH of the glycerol solution was at about the isoelectric point of hemoglobin. The data obtained are as follows:

P observed.	Capillarity.	Density.	P $cm.\ H_2O$	C	$P:C'$
5.86	0.48	1.135	6.12	1.55	3.95

Equilibrium was approached from the upper side; time of equilibrium, 14 days.

The molecular weight of hemoglobin in the glycerol solution may be calculated according to Equation 13, as follows:

$$M = \frac{C}{P} \cdot \frac{dRT}{100} = \frac{1}{3.95} \times 1.135 \times 2.32 \times 10^5 = 66{,}500$$

The value obtained is very close to the value for the molecular weight in aqueous solution and shows that glycerol produces no change in the state of the aggregation of hemoglobin. Hemoglobin is not denatured by solutions of glycerol. This strongly points to the altered molecular weight in urea solution being due to the denaturation.

Edestin.

The edestin used in the following experiments was kindly furnished to us by Professor C. L. A. Schmidt. It was prepared by him from hemp-seed by the method of Osborne (50). After crystallization, it was first washed a number of times with water in tall glass cylinders, then with alcohol, and finally with ether. The ash content was found to be 0.079 per cent. In the experimental work, the edestin solutions were analyzed by the same colorimetric method employed with casein. The standard was prepared by dissolving a known weight of edestin in 0.1 M NaOH.

Influence of pH on the Osmotic Pressure.

The solutions of edestin were, as in the previous work, prepared by dissolving the edestin in solutions of urea intended for the outer solutions (6.66 M urea, 0.05 M with respect to acetate or phosphate buffer of different pH values). When prepared in this way all of the edestin did not go into solution, so the solutions were centrifuged to remove the insoluble material present.

Edestin was found to have a minimum osmotic pressure over a rather wide range of hydrogen ion activity, pH 5.7 to 8.9, as is shown in Fig. 8. In solutions more acid than pH 5.7 the osmotic pressure increases quite rapidly. That this increase is due to a Donnan membrane equilibrium is shown qualitatively by the membrane potentials in the region of increasing osmotic pressure and practically zero membrane potential over the region of minimum osmotic pressure as determined by the difference in pH

between the inner and outer solutions. The data for this are given in Table IX.

The relationship between the osmotic pressure of edestin and the hydrogen ion activity is in harmony with the acid- and base-binding properties of this protein in aqueous solution. Hitchcock (51) has found that edestin shows no appreciable combination with acid or base over the pH range of 5 to 9 in aqueous solution. This range, allowing for a slight alkaline shift in the isoelectric

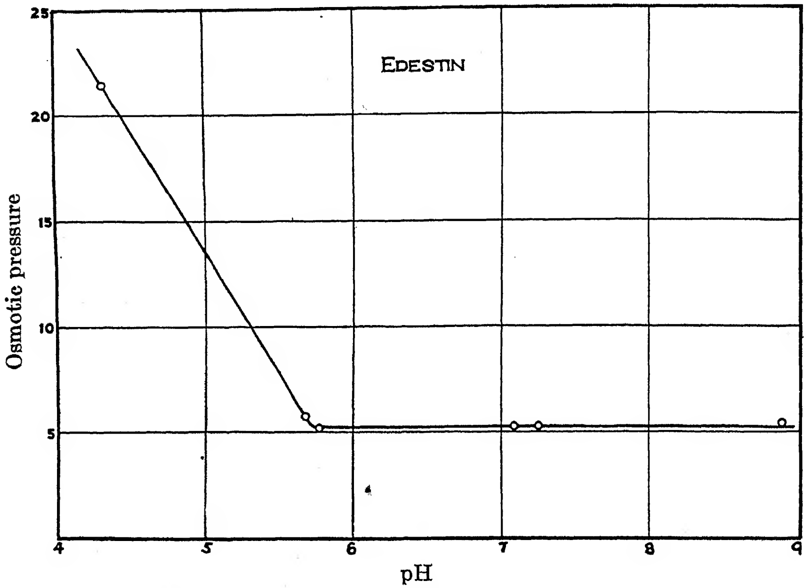

Fig. 8. Influence of the hydrogen ion activity on the osmotic pressure of edestin. Location of the hydrogen ion activity at which the osmotic pressure is a minimum. Ordinates, osmotic pressure of edestin per unit of concentration in cm. of water.

zone of edestin, corresponds to the region of constant minimum osmotic pressure.

Relationship between Osmotic Pressure and Concentration of Edestin.

Difficulty was encountered in preparing solutions of high concentration of edestin in the 6.66 M urea solutions, owing to the edestin becoming almost jelly-like in its consistency, and as a consequence osmotic pressure measurements at a greater concen-

tration than 3 gm. per 100 gm. of solvent were not obtained. The results obtained are shown graphically in Fig. 9, Curve 1.

TABLE IX.

Osmotic Pressure and pH Difference of Edestin.

C', per 100 gm. solvent.	P per unit concentration.	pH, inner solution.	pH, outer solution.	ΔpH
gm.	*cm.* H_2O			
0.499	21.44	4.32	4.10	+0.22
0.775	5.77	5.77	5.68	+0.09
0.690	5.17	5.78	5.76	+0.02
0.472	5.22	7.07	7.08	−0.01
0.842	5.47		8.87	

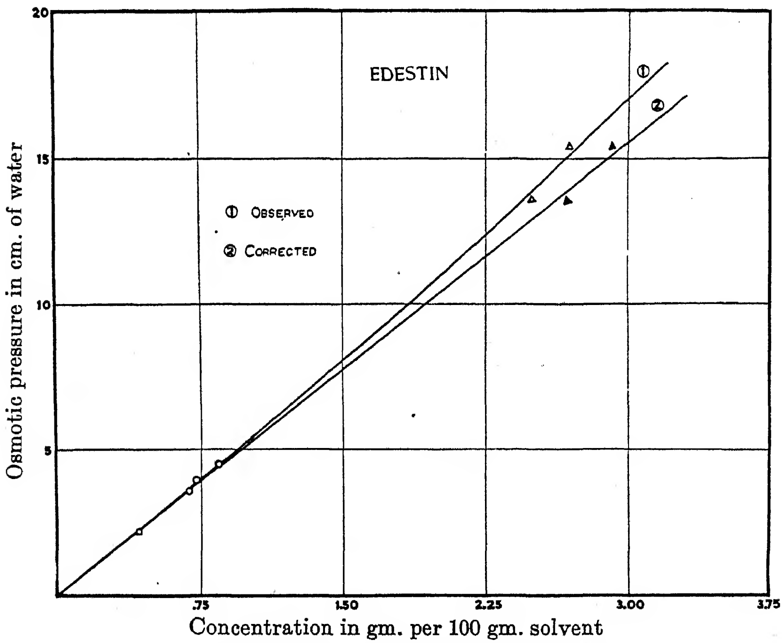

FIG. 9. Relationship between the osmotic pressure and the concentration of edestin at the isoelectric zone.

The measurements at higher concentrations show similarity to those upon casein and hemoglobin, and the osmotic pressure of

edestin increases somewhat more than is proportional to its concentration.

The concentration data for edestin, corrected by employing the value of 2.80 for the h term, follows the law of proportionality fairly well (Curve 2, Fig. 9) although here, because of the lower concentrations of edestin, it is not possible to determine this value with any degree of exactness.

It is interesting to note that with edestin, no buffering was required to obtain the correct value of osmotic pressure, as is illustrated by the first point of Curve 1, Fig. 9, which was carried out in unbuffered urea solution. This is evidence that the results obtained in buffered solution are not due to an aggregation effect of the salt of the buffer solution.

Molecular Weight of Edestin.

The molecular weight of edestin is calculated from the value of osmotic pressure which is a minimum with respect to hydrogen ion activity and which is corrected for solvation. The average value per unit of concentration obtained in this way, corresponding to the slope of Curve 2 in Fig. 9, is 5.14 cm. of water.

The molecular weight then is: $M = \frac{1}{5.14} \times 2.54 \times 10^5 =$ $49{,}500 \pm 700$.

Since this work was completed, a paper by Svedberg and Stamm (52) has appeared giving their results for the determination of the molecular weight of edestin by the ultracentrifuge method. The solvent they employed contained from 0.8 to 1 M NaCl. They found edestin to be homomolecular in composition and to have a molecular weight of $212{,}000 \pm 10{,}000$. It is striking that the value obtained by us is within the limits of error of measurement, one-fourth that found by Svedberg. Under certain conditions, Svedberg and Stamm found some evidence of the disintegration of edestin into smaller units, but not into fourths. If the strong salt solutions used by the above authors give the normal edestin and not an aggregated form, we must conclude that the urea solution produces a disaggregation of the edestin.

Osmotic Pressure and Molecular Weight of Egg Albumin.

A few measurements were made of the osmotic pressure of egg albumin solutions. This again was to obtain data on the influence of urea solution on a protein of known molecular weight.

The egg albumin was prepared by the method of Sørensen (35) as modified by La Rosa (53). It was crystallized three times from ammonium sulfate solution. The greater part of the ammonium sulfate was removed by dialysis for 12 hours against running tap water. In this way the conductivity was reduced to 4×10^{-4} mhos. The solution was next electrodialyzed against flowing distilled water to remove the last traces of electrolytes. After

TABLE X.

Molecular Weight of Egg Albumin.

	P observed.	Capillarity.	Density.	*P*	*C'*	*M*
	cm.	*cm.*		*cm.* H_2O		
Egg albumin in H_2O..................	2.60	0.45	1.00	2.05	0.333	35,000
" " " 6.66 M urea solution..	2.15	0.41	1.10	1.92	0.273	36,100
" " " 6.66 " " " ..	3.95	0.41	1.10	3.88	0.547	35,800

several hours of electrodialysis there was obtained a solution with a conductivity of 1.6×10^{-5} mhos.

One determination was carried out in water solution buffered with 0.0275 M acetate, adjusted to pH 4.8, the isoelectric point of egg albumin. This determination was made as a check on our experimental technique. The solutions in the experiments with 6.66 M urea were adjusted also to pH 4.8 with acetate buffer at 0.05 M concentration as in our previous experiments. It was assumed, since the isoelectric point of egg albumin is not sharp (cataphoretic measurements by Michaelis and Davidsohn (54) give pH 4.44 to 4.96), that a moderate shift in the isoelectric region could occur in urea solution and would still allow the pH of 4.8 to fall within its limits.

The results are given in Table X. Not much stress is laid upon the accuracy of the absolute values obtained for the molecular

weight of egg albumin, since the solutions employed were quite dilute. However, the value obtained in aqueous solution agrees within 6 per cent of the value obtained by Sørensen (35) and Svedberg and Nichols (55). Of great interest is the finding that there is no difference between the molecular weight in water and in urea solution. Egg albumin is denatured by solutions of urea as is hemoglobin (45). It is rather difficult to demonstrate this and it is only by dialyzing the solution free of urea that the insoluble egg albumin is obtained. It is of interest that in contrast to hemoglobin, denaturation results in no change in the size of the albumin molecule.

Concluding Remarks.—The molecular weights of a large number of proteins, calculated from the content of their various constituents, the amino acids, phosphorus, sulfur, etc., have been exhaustively appraised by Cohn, Hendry, and Prentiss (56). In Table

TABLE XI.

Molecular Weight Comparisons.

	Minimum molecular weight, chemical data.	Molecular weight in aqueous solution.	Molecular weight, osmotic pressure in urea solutions.
Casein	12,800	*	33,600
Edestin	29,000	212,000	49,500
Hemoglobin	16,660	67,000	34,300
Egg albumin	33,800	34,000	36,000

* The values 75,000 to 100,000 are given in a recent paper by Svedberg, Carpenter, and Carpenter (57), which came to our attention while this article was in press. They refer to the molecular weight of "the bulk of the casein prepared by Van Slyke and Baker's method." A somewhat higher value, 375,000, was obtained by them (58) upon an acid-alcohol-soluble fraction of Hammarsten casein.

XI there are given a comparison for the proteins studied by us and of the values calculated from chemical data, measurements by the ultracentrifuge and osmotic pressure in aqueous solution, and from measurements in urea solution. It is to be observed that only for egg albumin and hemoglobin can the data from chemical analysis

be brought into accord with the actually determined molecular weights.[8]

The molecular weight obtained for edestin in urea solution is also not in accord with a theory of protein structure proposed by Svedberg (52). It is his contention that all the proteins are built up of elementary units having a molecular weight of 33,500. The figures obtained for edestin by us cannot be brought into accord with this hypothesis. A unit of half the molecular weight given by Svedberg, approximately 16,500, is required to be able to fit edestin into the scheme.

The original data are on file in the University of California Library, Berkeley.

We wish to acknowledge our indebtedness to Professor C. L. A. Schmidt for helpful suggestions and to Professor J. P. Bennet, of the Division of Pomology for the use of a low temperature room.

SUMMARY.

1. A method has been developed for the determination of the molecular weights of proteins that are not soluble in water. It depends upon the measurement of the osmotic pressure, in an isoelectric condition, of the proteins dissolved in certain organic solvents, particularly urea-water solutions.

2. A solution of urea in water as a solvent has a depressing action on the ionization of electrolytes. The apparent dissociation constant pK' of acetic acid is reduced to 5.25 and the pK_2' of phosphoric acid to 7.22.

3. The osmotic pressure of casein, edestin, and hemoglobin was found to increase to more than is proportional to the increase of concentration. The curves for these proteins can be corrected for this deviation from proportionality by the use of the

[8] It has been pointed out to us that the value we obtained for the molecular weight of casein in urea solutions is in accord with data from chemical analysis considered from one standpoint; namely, that "the weight of casein that contains 1 atom of sulfide sulfur calculated from Osborne's analysis is 31,752. The concordance between this result and the molecular weight in urea solutions is suggestive of a possible state of casein under these conditions." (The value of 12,800 for the minimum molecular weight of casein given in Table XI rests largely upon the tryptophane content (56).)

equation $C = \frac{100\,C'}{100 - hC'}$, in which C' is the measured concentration and h is a constant. A value of 2.80 for h was found to make the corrected concentration, C, proportional to the osmotic pressure for all three proteins. If the deviation from proportionality is due to a solvation of the proteins, then 2.80 gm. of solvent per gm. of protein are a measure of the solvation.

4. From the osmotic pressure measurements in urea solutions, controlled with respect to hydrogen ion activity and corrected for the deviation from proportionality, the molecular weights of the following proteins were calculated as follows: casein 33,600 ± 250; edestin 49,500 ± 700; hemoglobin 34,300 ± 425; and egg albumin 36,000.

5. The change of solvent has been found to cause a change in the state of aggregation of hemoglobin. Dissolved in urea solution it has half the molecular weight found in water solution. On the other hand the molecular weight of egg albumin is not changed by the urea. Both hemoglobin and egg albumin are denatured by urea solutions, the hemoglobin being converted to cathemoglobin.

6. Measured in 6.5 M glycerol solution, hemoglobin has the same molecular weight as in aqueous solution. In glycerol solution hemoglobin is not denatured.

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GLUCOSE TOLERANCE IN AVITAMINOSIS DUE TO LOW ANTINEURITIC VITAMIN B.*

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INTRODUCTION.

As early as 1914 Funk (1) related carbohydrate metabolism to beriberi in the pigeon. The basis for this was the more rapid production of polyneuritis on "carbohydrate-rich diets." Shortly after, he and von Schönborn (2) reported hyperglycemia with accompanying shortage of liver glycogen as further evidence for this view.

McCarrison (3) in 1919 reported the hypertrophy of the adrenals and an acidosis in vitamin B deficiency, which he associated with imperfect metabolism of carbohydrates; and this view was supported by Magne and Simonnet (4) who stated that an injection of glucose failed to raise the respiratory quotient of the animal with beriberi, but did in cases of inanition.

Mattill (5) concluded from a study of the R.Q. of the rat that no interference of glucose combustion was traceable to vitamin B deficiency. He also mentioned, however, that a slower rise in the R.Q. (1 to 3 hours) occurred in the glucose-fed animals deficient in vitamin B; some of the animals prostrated by the glucose did not survive.

Eggleton and Gross (6) failed to find any marked hyperglycemia or any difference in glucose tolerance, but reported gradual depletion of liver glycogen which they ascribed to inanition.

Drummond and Marrian (7) have related vitamin B deficiency

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to inanition, and among other evidence to support such a claim, they state the following:

> "In both conditions [*i.e.*, inanition and vitamin B deficiency] a state of hyperglycæmia develops—associated with a hypertrophy of the adrenal glands—but is followed by a sharp fall to values much below the normal level. The period during which this reduction occurs is that characterised by the fall of body temperature and oxygen consumption.
>
> These results are discussed in the light of existing theories regarding the physiological action of vitamin B, and the conclusion is reached that, in the case of rats at any rate, the nutritive failure following a deficiency of the vitamin is virtually identical with that resulting from starvation."

Recent work in this laboratory strongly suggests that vitamin B must be in some way related to carbohydrate metabolism. It has been found here (8) that when fat forms the sole source of non-protein energy in a diet, much less vitamin B is necessary for growth and well being than when sucrose is the sole source of such energy, but that when a high level of vitamin B is fed the sucrose diet can hardly be called disadvantageous. We are thus forced to assign to vitamin B properties which enable the animal organism better to utilize sucrose. We felt a reinvestigation of the relation of vitamin B to carbohydrate metabolism to be highly desirable.

Plan of Experiment.

We chose the glucose tolerance test to investigate any disturbance in carbohydrate metabolism due to low vitamin B. We were aware of the negative results reported by Eggleton and Gross (6); but we felt that the glucose tolerance test was still the best method of approach to this problem because of its sensitivity to disturbance in carbohydrate metabolism. Our technique differed from that of Eggleton and Gross in the following ways: (1) instead of killing a rat for each point on the glucose tolerance curve, we used the same animal for *all* the points in the curve; (2) our animals were definitely more avitaminotic than those of Eggleton and Gross because their diets carried starch and fat, and these have a definite sparing action on vitamin B (8). Furthermore, they fed lemon juice to their animals, and lemon juice carries considerable vitamin B.

Methods.

21 day old female rats were placed on our Diet 540[1] (free from both of the B vitamins) for a period of 10 days—each receiving 2 drops of cod liver oil (Patch) daily. At this time they were placed on our Diet 542, which is the same as Diet 540 save for 10 per cent added autoclaved yeast to supply the heat-stable water-soluble vitamin.[2] The yeast replaced an equivalent weight of sugar with such changes in casein as were necessary to keep the nutritive ratio constant. In addition, each animal was fed 0.30 gm. of yeast known from previous test to be poor in the antineuritic factor. After about 3 weeks on this dietary regimen, the body weight curve of the animals had reached a plateau at about 50 to 60 gm. At this time the animals were selected in pairs of about equal weight. Each animal was placed in an individual wire bottom cage in a room where the temperature was held at 20° by thermostatic control. One of each pair had its yeast of poor quality replaced by yeast known to be potent in vitamin B. Thus two similar diets were used with the only known difference being their content of vitamin B. The animal getting the yeast of good quality had a much greater appetite than the animal receiving the yeast of poor quality; therefore, in order to keep the food intake the same for both animals the food eaten by the animal receiving the low vitamin B diet was weighed daily and a similar amount of food was fed its more hungry control. This procedure was followed wherever possible.

Glucose Tolerance Test.

For glucose tolerance tests the animals were fasted for 12 to 15 hours. They were allowed free access to water. The fasting blood sugar level was determined and glucose solution (Pfanstiehl) was immediately given by stomach tube. Blood sugar determinations were then made at 15, 30, 60, and 120 minute periods. The blood sugar levels were then plotted against time as blood sugar tolerance curves. The first point on each of these curves represents the fasting blood sugar level.

[1] Diet 540 contains casein (extracted with acidulated water for 1 week and finally dehydrated with alcohol and ether) 25 parts, commercial cane sugar 75 parts, salts (McCollum No. 185 (9)) 4 parts.

[2] This vitamin is now commonly referred to as vitamin G in this country and vitamin B_2 in England.

1 gm. of glucose per 100 gm. of body weight was fed in a 50 per cent solution by stomach tube (a No. 6 urethral catheter attached to a hypodermic serving the purpose well). The animal's mouth was held open by a wire spring slipped over the upper and lower teeth. The stomach tube was then easily slid through the esophagus into the stomach with the animal breathing normally and with little inconvenience. This is essentially the procedure described by Cori (10).

Method of Sampling and Chemical Procedure.

During the glucose tolerance test period the rats were kept in a wire bottom, temperature-controlled (27°) chamber. The rat's tail was placed in a large beaker of water at 39° for 1 minute. It was quickly blotted dry with clean filter paper and then laid on another clean filter paper. The very tip end of the tail was cut with a sharp scalpel, making a clean cut. The freely flowing blood was allowed to drain on a clean paraffin block until slightly over 0.10 cc. was obtained. The tail was immediately dipped in melted paraffin to stop the bleeding. It was then wrapped with thread at the tip and then the thread was sealed with paraffin. With this treatment the animals experienced little or no discomfort.

The 0.10 cc. of freshly drawn blood was immediately measured in a clean, dry, accurately calibrated capillary pipette, and was placed in exactly 10 cc. of *freshly prepared* tungstic acid before the blood clotted. The reducing value was obtained in the next few hours by Folin's method (11).

Results of Glucose Tolerance Experiments.

The data leave little doubt that the avitaminotic animal has some disturbance in its carbohydrate metabolism as indicated by glucose tolerance curves. There existed the possibility of a greater concentration of blood in the avitaminotic animal than in its control, which might cause the higher blood sugar values. This possibility led us to investigate the specific gravity under the particular conditions used in the glucose tolerance test. The results are presented in Chart I. Animals low in vitamin B and the controls (also shown in Chart III) were fasted, fed glucose, and bled under the same conditions and intervals as outlined for the glucose tolerance test. The specific gravity was immediately

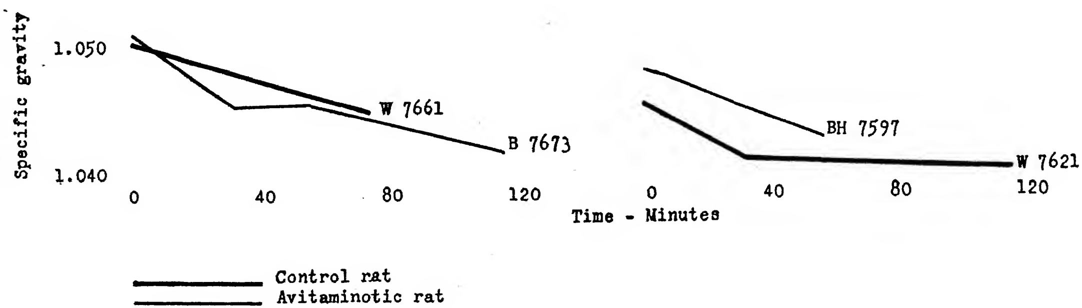

CHART I. Effect of the administration of glucose on the specific gravity of the blood. Dose: 2 cc. of 50 per cent solution of glucose per 100 gm. of body weight given immediately after the first blood sample was taken.

determined by the method of Barbour (12), and is represented plotted against time. That a gradual decrease in specific gravity occurred in the avitaminotic animals as well as in their controls shows that the high blood sugar levels were not influenced by a concentration of the blood. It is also to be noted that no essential difference in specific gravity was obtained between the animals low in vitamin B and their controls.

Inasmuch as there is bound to be a certain amount of excitement during glucose feeding and bleeding, it was deemed desirable to find out the effect such handling of the animals had upon their

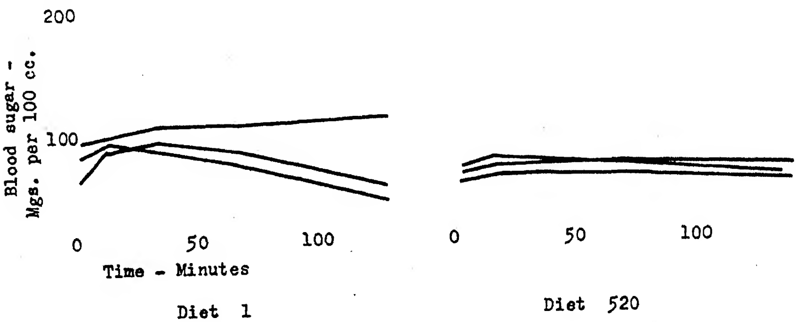

CHART II. Blood sugar curves of animals maintained on a natural food (Diet 1) and of animals maintained on a synthetic ration (Diet 520), each group receiving 2 cc. per 100 gm. of body weight of distilled water in place of an equal amount of 50 per cent solution of glucose. Diet 1: whole wheat 67.5, casein 15, milk powder 10, sodium chloride 1, calcium carbonate 1.5, butter fat 5. Diet 520: casein (commercial) 27.5, sucrose 75, salts (McCollum No. 185) 4, supplemented with 0.7 gm. of yeast known to be low in antineuritic vitamin B and 2 drops of cod liver oil daily.

glucose tolerance curves. Such curves are presented in Chart II. The animals used for this test comprised a group of animals on a normal diet composed of natural foodstuffs, and a synthetic diet low in vitamin B but not so rigorously deficient in this vitamin as our Diet 540. It can readily be seen that while a small error creeps into the glucose tolerance curve, it is not appreciable, and what disturbance does occur seems greater in the normal animals than in those suffering from vitamin B deficiency.

Chart III shows some typical glucose tolerance curves obtained in these studies. The animals were definitely avitaminotic, but

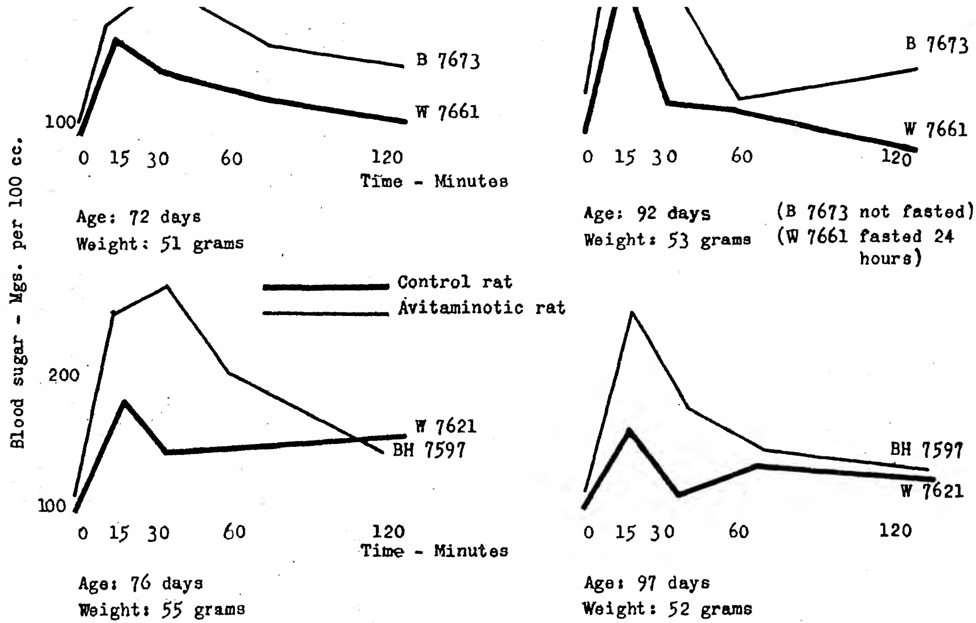

Chart III. Glucose tolerance curves of animals maintained on a diet low in vitamin B and the glucose tolerance curves of their controls receiving the same amount of the same diet (Diet 542) but receiving in addition an adequate amount of vitamin B. The animals in the first group received 300 mg. daily of yeast known to be low in vitamin B and those in the control group received 300 mg. of yeast known to be rich in this factor. The avitaminotic rats used here showed no symptoms of beriberi.

none of them showed signs of beriberi. The controls were identical in every respect to the avitaminotic animals with the exception that their food intake included vitamin B.

Chart IV shows an animal definitely avitaminotic, as seen from its limited growth, but in rather good condition, and its control. At 118 and 145 days of age, the avitaminotic animal shows but a slightly poorer tolerance curve than its control. The glucose tolerance here is definitely better than that displayed in Chart III and it should be correlated with a milder degree of avitaminosis due to slightly greater intake of vitamin B.

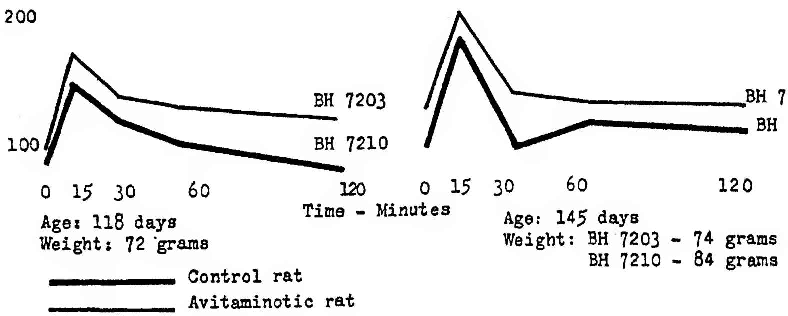

CHART IV. Glucose tolerance curves of a rat when 118 and 145 days old. This rat was maintained on Diet 542 plus 1.0 gm. of yeast known to be low in vitamin B, so that the animal, while not rigorously avitaminotic, was definitely stunted due to low vitamin B intake. The control rat received the same amount by weight of Diet 542 and in addition 1.0 gm. of yeast known to be rich in vitamin B.

In Charts V and VI are presented glucose tolerance curves of avitaminotic animals and their controls when in advanced stages of vitamin B avitaminosis or actually with beriberi. These curves leave no doubt that in this stage of vitamin B avitaminosis there is a definite interference in the metabolism of carbohydrates in rats.

In Chart V it will be seen that while some of the controls show a much greater tolerance to glucose than the avitaminotic animals, they exhibit a poorer tolerance than the controls shown in Charts III and IV, the latter group being representative of numerous other similar experiments. The only explanation we can offer

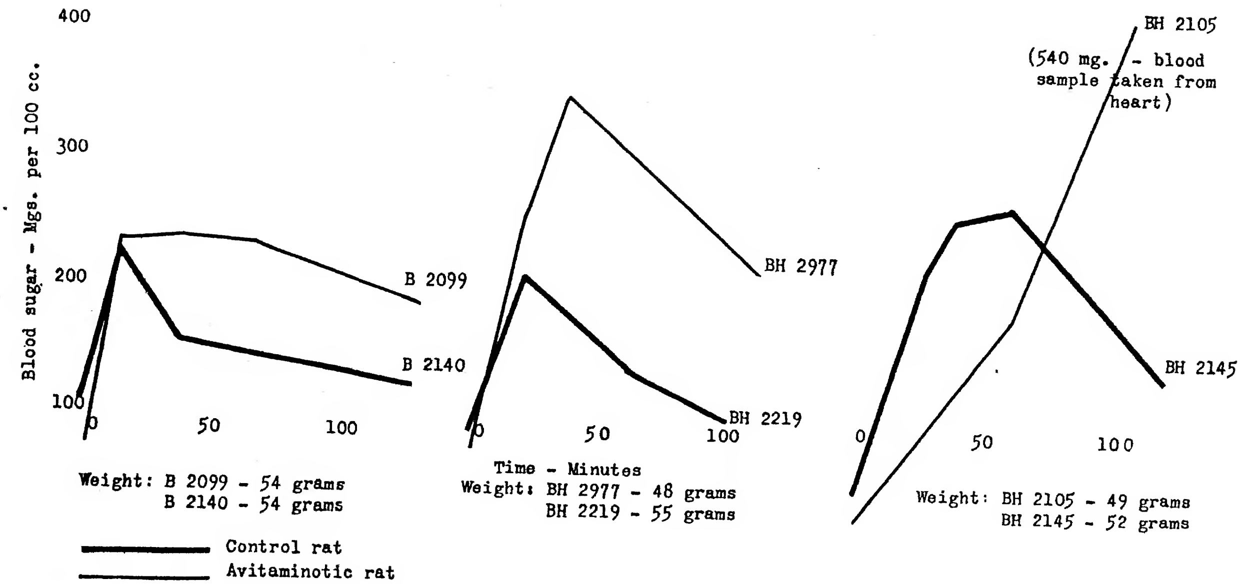

CHART V. Glucose tolerance curves of rats with beriberi together with those of controls protected from beriberi by the addition to their diet of yeast known to be rich in vitamin B. Both groups were reared on Diet 542 fed *ad libitum* plus 0.5 gm. of yeast low in vitamin B. From 7 to 16 days before the test, the control group received the same amount of diet by weight as the experimental group, plus 0.5 gm. of yeast known to be rich in vitamin B (Rat B 2140, 7 days; Rat BH 2145, 9 days; Rat BH 2219, 16 days).

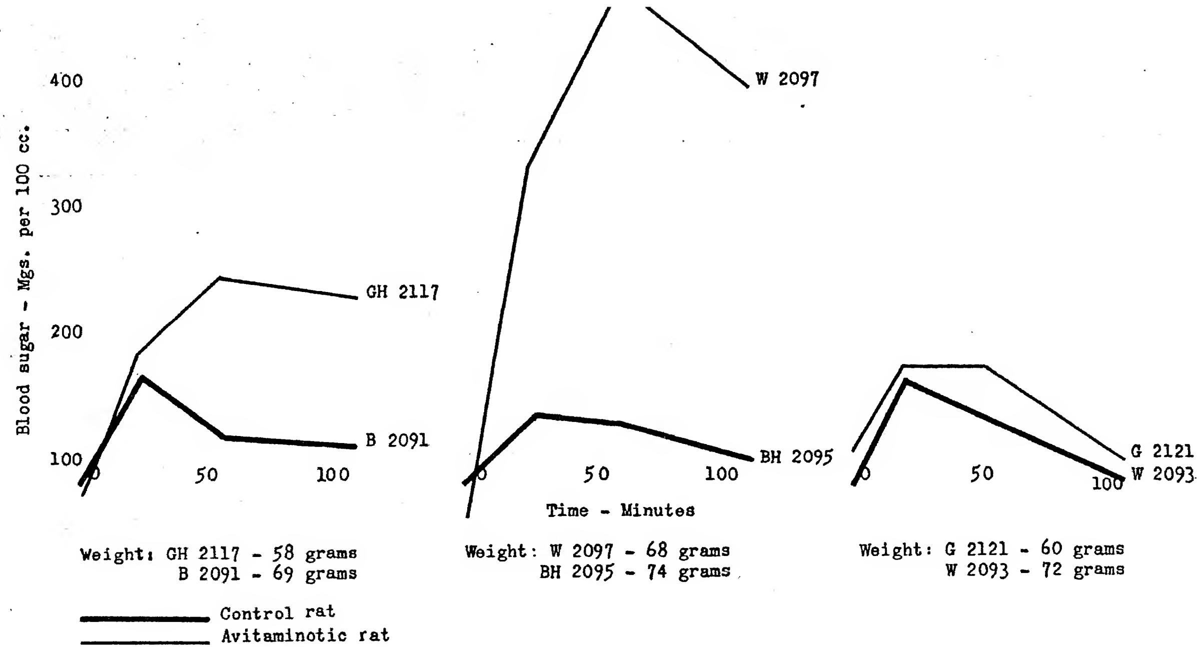

CHART VI. Glucose tolerance curves of rats at the time of the first symptoms of beriberi, together with the glucose tolerance curves of controls receiving adequate vitamin B. Both groups of animals were reared on Diet 542 fed *ad libitum* plus 0.5 gm. of yeast low in vitamin B. From 8 to 11 days before the test the control group received the same amount by weight of Diet 542 as the experimental group and in addition had the poor yeast replaced by yeast rich in vitamin B (Rat BH 2095, 8 days; Rat B 2091, 11 days; Rat W 2093, 11 days).

for this decrease in the tolerance of the controls is the short period of time during which the vitamin therapy was administered to the animals. It seems that close to 3 weeks of vitamin therapy are sometimes necessary before the tolerance to glucose comes back to normal. In the chart under discussion (Chart V) the controls received vitamin therapy for a much shorter period of time than 3 weeks. The control rats given in Chart VI show a greater tolerance to glucose than those in Chart V, yet they had received vitamin therapy for about the same period of time. In this connection it will be noticed that the control animals shown in Chart VI are appreciably heavier than those in Chart V, and this may possibly have something to do with their greater response to vitamin B therapy.

Although experience from other work indicated that carbohydrate metabolism in vitamin B avitaminosis may be a primary disturbance (8), it must be admitted that the particular disturbance in carbohydrate metabolism which becomes evident in a decreased glucose tolerance is late in appearing, and is possibly of a secondary nature.

The glucose tolerance curves leave little doubt that there is a final disturbance in the carbohydrate mechanism of rats rendered avitaminotic due to low vitamin B intake. But it is remarkable that for a considerable time animals may be definitely avitaminotic as evidenced by (1) plateau in weight and (2) marked response to vitamin B therapy and yet give glucose tolerance curves nearly identical with those of their controls. Sooner or later the administration of glucose results in a blood sugar curve which is higher and remains higher than in controls identical in every way experimentally possible. The most marked disturbance in glucose tolerance always occurs in the premortal stage. In clearly expressed beriberi, which is the extreme form of vitamin B disturbance, the animals show little or no tolerance for glucose, the blood sugar often rising steeply without showing any tendency to go down after 2 hours. The rapid ascent which occurs in most cases demonstrates, of course, that the permeability of the intestine is not adversely affected. We studied our controls at the same time as the avitaminotic animals, hoping in this way to know the glucose tolerance in both groups at the same stage. But we must leave open the question of whether we actually

succeeded in this attempt. It is quite possible that, in spite of every effort and precaution, our control rats may not have been actually so near their death as were the animals with beriberi.

SUMMARY.

1. A method is described for determining the glucose tolerance of the rat without killing the animal.

2. Animals suffer for a long period from vitamin B deficiency without appreciable lowering of their glucose tolerance.

3. But it is true that animals approaching their final breakdown from beriberi exhibit a poorer glucose tolerance than their controls which have been maintained on an identical food intake but including adequate vitamin B.

4. The poorest tolerance is exhibited by rats showing typical signs of beriberi or when near that stage. As the avitaminosis decreases, the glucose tolerance increases, until a stage in avitaminosis can again be reached which does not interfere markedly with glucose tolerance.

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A PROTEOLYTIC ENZYME IN FICIN, THE ANTHELMINTIC PRINCIPLE OF LECHE DE HIGUERON.*

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Leche de higueron is the sap of the *Ficus laurifolia* (1, 2), a tree which is very closely related to the domestic fig tree.[1] It has been widely used as a general anthelmintic by the natives of South America and Panama and its anthelmintic action confirmed by several medical investigators who have noticed that it was particularly effective against *Trichuris trichiura.* The most recent work on this subject is that of Dr. Caldwell (4) of the International Health Board stationed in Andalusia, Alabama, who kindly furnished us with the material which we have used in these

* The funds for carrying out this work were given by the International Health Division of the Rockefeller Foundation.

[1] Relation of rubber trees to fig trees.

Order	Family.	Genus.	Species.
Urticales	Euphorbiaceæ	*Hevea*	*Hevea braziliensis* (best source of rubber)
	Moraceæ	*Ficus*	*Ficus elastica*
			" *anthelmintica*
			" *laurifolia*†
			" *doliaria*
			" *glabrata*†
			" *carica,* L. (domestic fig tree)

† There is some difference of opinion as to which of these two species is the source of the leche de higueron. Hall and Augustine (2) and Chandler (1), say that leche de higueron comes from the *Ficus laurifolia,* whereas Paez (3) says the leche de higueron is obtained from the latex of the *Ficus glabrata.*

experiments to determine the nature of the active principle and its mode of action.[2]

If ascaris are placed in Ringer's solution containing 1 to 2 per cent of leche de higueron they soon become wrinkled and flaccid, with the development in 1 to 2 hours of small blisters or ulcers, which later perforate and cause death. This simple criterion of activity furnished us with a method of fractionating the sap and tracing the active principle. The ascaris used were obtained fresh from the intestines of pigs within 30 minutes after the pigs were slaughtered. The ascaris were placed immediately in a thermos jug containing Ringer's solution at 38° and brought to the laboratory where they were placed in fresh Ringer's solution in an incubator at 36°. The experiments reported were carried out on the ascaris during the first 2 or 3 days after their collection. Brown (5) has shown in some recent experiments that the ascaris can be kept in Ringer's solution for some 10 to 15 days without an appreciable change in their activity or metabolism.

Purification of Sap.

The crude sap, which is a yellow-white syrup, was found to contain about 25 per cent by weight of a protein-like material in suspension. 40 per cent of the suspended matter separates out on centrifuging and is non-active. The fluid portion holds in suspension an amorphous substance which precipitates out with protein precipitants, forming a flocculent precipitate with $HgCl_2$ or $MgSO_4$, or a waxy mass when 3 parts of acetone or 5 parts of alcohol are added. This precipitate contains the active principle. By redissolving the acetone precipitate in water and reprecipitating with acetone, then washing with acetone and drying in a vacuum dessicator with $CaCl_2$, a light yellowish powder is obtained, which is quite active. From 100 cc. of the sap one obtains about 11 to 12 gm. of the powder. The name ficin, from

[2] This leche de higueron that we received from Dr. Caldwell was obtained originally from a native physician, Dr. M. A. Villalobos, in Santa Marta, Colombia. Further work is being done to establish just what species of *Ficus* contain the active principle. We were unable to determine the species from which the material that we studied was obtained. A fresh sample of leche de higueron which was collected from the *Ficus glabrata* has just been received by us; preliminary experiments indicate it to have the same action as the material obtained from Dr. Villalobos.

Ficus, the generic name of the tree from which leche de higueron is obtained, has been given by us to this purified protein powder containing the active anthelmintic principle. Upon testing this precipitate for free amino groups and for organic sulfur, both were found present. The nitrogen content was 16 per cent, while the sulfur was 1.05 per cent. These values are well within the normal ranges for proteins. The color tests for proteins and various units were positive; that is, the precipitate with biuret, xanthoproteic, Millon's, and Heller's tests gave typical results for proteins. We do not wish to leave the impression that we believe the precipitate to be the pure principle or that the active part is necessarily of protein nature but that the active principle is contained in the precipitated protein mixture.

Activity of Concentrated Material.

The action of this purified protein precipitate, ficin, on ascaris is shown in the following experiment: A 0.1 to 0.2 per cent solution of ficin was made in Ringer's solution. Fresh ascaris (two to ten) were placed in a flask containing 100 cc. of the solution, and incubated at 37°. In 1 hour the worms become wrinkled, flaccid, and may show slight blister-like lesions at the anterior end. After $1\frac{1}{2}$ hours the lesions change to large ulcers 1×3 mm. which can be seen over the body, extending almost through the body wall. At 2 hours the lesions have penetrated the whole thickness of the body wall and the viscera (ovaries, oviduct, uterus, and intestine) may be seen protruding through the opening. Although the worms are in a very mutilated state they are still able to move around in the fluid. On further digestion the whole external surface of the worm becomes ulcerated and goes into solution.

Temperature of Inactivation.

The effect of temperature was then determined on the activity of ficin. 50 cc. portions of a 0.3 per cent solution of the protein precipitate in Ringer's solution were heated for 5 minutes at the following temperatures, 40°, 50°, 55°, 60°, 65°, 70°, 75°, 80°, 90°, 97°, and then cooled and incubated at 35°. Ascarids placed in these solutions were digested at the end of 2 hours in the solutions that had been heated to 70° or below, whereas those in the solutions heated to 75° or above were not changed at the end of 5 hours (Fig. 1).

Type of Proteolytic Enzyme.

These experientsm showing that the active principle was contained in a substance of protein nature, easily destroyed by heat, and causing lesions of a digestive type suggested that we were

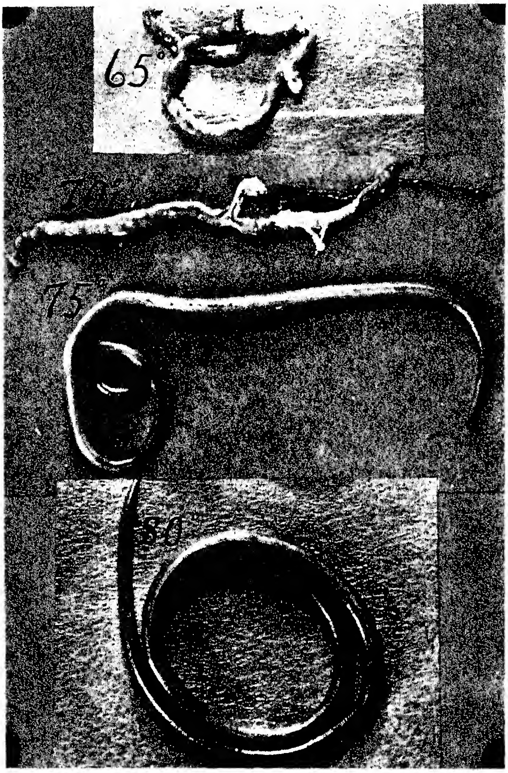

FIG. 1. A comparison of ascaris after 2 hours incubation in 0.2 per cent solutions of the active precipitate which had been heated to temperatures of 65°, 70°, 75°, and 80° for 5 minutes. A temperature of 75° for 5 minutes destroys the digestive action of the material on the ascaris.

dealing with an enzyme, which was confirmed by the following experiments.

Experiment 1. Hydrolysis of Coagulated Egg White in Mett's Tubes.—The coagulated egg albumin was digested for an average length of 8 mm. in 18 hours by a 0.2 per cent solution of ficin;

this indicated a proteolytic enzyme of the pepsin or trypsin type.

Experiment 2. Hydrolysis of Casein.

(*a*) The hydrolysis of casein was shown by an increase in the formol titration for free carboxyl groups. A 4 per cent casein suspension was digested with 0.4 per cent ficin for 5 hours at 35°. The results of formol titration at the beginning and end of digestion are as follows:

	0.1 N NaOH per 100 cc. mixture.
	cc.
Original titration	23
After 5 hours digestion	50
Increase	27

The figures show that the free carboxyl radicals were increased over 100 per cent in 5 hours digestion (proteolytic enzyme, pepsin or trypsin).

(*b*) A digestion mixture was made containing 0.4 per cent ficin and 10 per cent casein, with toluene as a preservative. This mixture was digested for 4 days at 35°; at the end of this time a test for free tryptophane was positive. On concentrating the fluid (after heat coagulation, neutralization, and filtration) and placing in an ice box for 12 hours, crystals of pure tyrosine and "leucine balls" (tyrosine + leucine) separated out, showing digestion of the casein down to the amino acids (trypsin or pepsin + erepsin).

Optimum Hydrogen Ion Concentration.

The activity of the enzyme at various hydrogen ion concentrations was determined. A 0.2 per cent solution of ficin in Ringer's solution was made, and a series of 50 cc. portions adjusted, by the addition of dilute HCl or NaOH, to hydrogen ion concentrations varying from pH 2 to 9. Fresh ascaris were placed in these solutions and incubated at 37°. It was found that at pH 2 to 3 the enzymatic activity was destroyed, because it could not be brought back by neutralization. This may explain why such an enormous anthelmintic dose of the crude material is given (60 cc. of sap = 8 gm. of powder) as a large portion may be destroyed on passing through the stomach at a pH of 2 to 3. This indicates that the enzyme is not of the pepsin class because

it is destroyed or retarded by high acid concentration, pH 2 to 3. Throughout the pH range of 4 to 8.5 the enzyme is active. The enzyme must therefore be of the tryptic class; as, however, the proteolytic enzymes, pepsin and trypsin, have no action on ascaris, and papain only a slight action (6), this enzyme from leche de higueron must differ from these well known substances. Mendel and Blood (7) in a study on the proteolysis by papain and other proteolytic enzymes showed that there were in the saline extract of the ascaris antipeptic and antitryptic enzymes, but that there was not an antienzyme of papain present. These plant enzymes, papain and that in ficin, may have some peculiar property or mode of attack which enables them to act on the living organism. Ficin was found to digest hookworm, but less readily than ascaris and had no effect on the spiny headed worm which is found living together with ascaris in the pig's intestine.

Possible Presence of More Than One Enzyme.

Because of the unusual ability of leche de higueron to attack the living organism, it was suggested that there might be two agents present, one which killed the tissue, and another which caused digestion of the dead material. We have tried to determine this point by the following experiments, in which the worms were incubated in: (1) 0.3 per cent solution of ficin; (2) 0.3 per cent solution of ficin which had been heated for 5 minutes at 75°; (3) 0.3 per cent solution of ficin which had been heated for 5 minutes at 75° and to which trypsin was added after heating (0.5 gm. per 100 cc.); (4) 0.3 per cent solution of ficin for 15 minutes and then in 0.5 per cent trypsin solution; (5) 0.5 per cent trypsin; (6) 0.5 per cent pepsin. It was found that Solution 1 (0.3 per cent ficin) was the only one to digest the ascaris; this shows that if two agents are present both are destroyed by a temperature of 75° for 5 minutes, or that trypsin cannot replace the action of the proteolytic enzyme in ficin on ascaris, or that the agent which kills the tissue, if there is one, so that the proteolytic enzyme can act is destroyed at that temperature. The available data on this active material are insufficient to show whether or not there is only one or more than one active agent. Further work is being done on this question with hope of determining the number of agents present.

Further experiments are being carried out on the effect of this

enzyme on the gastrointestinal tract and on any secondary results from its proteolytic action when given by mouth.

CONCLUSION.

1. The active principle of leche de higueron is completely precipitated from aqueous solution by 3 parts of acetone or 5 parts of alcohol.

2. The active precipitate, ficin, digests ascaris in a dilution of 0.1 per cent in $1\frac{1}{2}$ to 2 hours.

3. The activity is destroyed by heating the aqueous solution for 5 minutes at 75°.

4. The precipitate will hydrolyze coagulated egg albumin and digest casein to the amino acid stage.

5. The activity is destroyed in $1\frac{1}{2}$ hours at pH 2 to 3.

These data show the proteolytic enzyme of the ascaris-dissolving principle in leche de higueron to be of the tryptic type.

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FACTORS DETERMINING THE ERGOSTEROL CONTENT OF YEAST.

I. SPECIES.*

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Following the discovery of ergosterol by Tanret (1), Gérard (2) observed that not only ergot, but fungi in general, contain it. Gérard was the first to recognize ergosterol in yeast, although Nägeli (3) had earlier obtained a yeast "cholesterol" in crude form. In traces ergosterol is now known to be widely distributed in both plant and animal kingdoms, but the fungi, and particularly the yeasts, remain its practical source.

Few studies have been reported on the factors which determine the ergosterol content of yeast. MacLean and Hoffert (4) noted differences in the sterol content of different samples, and MacLean (5) increased the sterol content of a given yeast by incubation in a phosphate-carbohydrate solution. Heiduschka and Lindner (6) compared the ergosterol content of ten fungi, including yeasts, finding from 0.29 to 1.17 per cent of ergosterol in the dry substance. They observed that the ergosterol content was influenced considerably by the composition of the wort. Bills and Cox (7) reported that one bakers' yeast yielded 10 times as much ergosterol as another, and that the two specimens of ergosterol were not identical, but more or less isomeric.

In 1927 it was observed that the bakers' yeast which we were attempting to use as a source of commercial ergosterol varied greatly in sterol content. Since care is exercised in the manu-

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facture of yeast to keep it uniform, it seemed that the ergosterol content must be extraordinarily sensitive to the conditions of culture. This supposition was supported by a later observation that another yeast, obtained fresh from a brewery, contained scarcely a trace of ergosterol; yet this same yeast, grown in an aerated wort, became an excellent source.

Our plan of investigation has been to culture in a given medium a variety of yeasts, and to select from these the most promising species for studies upon the influence of cultural conditions. It has been our purpose not only to develop commercial ergosterol production, but also to study the significance of ergosterol in the metabolism of fungi.

Cultivation and Harvest.

Twenty-nine cultures were obtained for study. They were carried on 1 per cent dextrose-nutrient agar slants. Each inoculum was prepared by washing a 24 hour growth from a slant into 50 cc. of wort broth, which was then incubated for 24 hours at 26–28°. Each inoculum, after being examined microscopically for freedom from bacterial contamination, was transferred to a 6 liter balloon flask containing 1 liter of culture medium. An aeration tube was extended through the cotton stopper of each flask into the medium. A sterile cotton filter was connected between the aeration tube and the compressed air line. A fine adjustment was provided to regulate the flow of air. The flasks were set up in batteries of twelve.

Ingredients for 12 liters of medium were dissolved, made up to volume, and divided among twelve flasks. The cotton stoppers and aeration tubes were inserted, and the whole set-up was then sterilized for 20 minutes under 20 pounds steam pressure. The heating caused a precipitate, presumably of phosphate, but this was not filtered out. In every experiment the medium had the following composition.

Final beet molasses	168	gm.
Urea	7.0	"
KH_2PO_4	3.0	"
$MgSO_4 \cdot 7H_2O$	0.5	"
$CaCl_2$	0.1	"
Water, *q.s.*	1000	cc.

TABLE I.

Ergosterol Content of Different Yeasts.

Species or description of yeast.	Guilliermond-Tanner classification. Saccharomycetaceæ. Group.	Genus.	American Type Culture Collection culture No.	Yield of dry yeast per liter of medium.	Yield of ergosterol per liter of medium.	Ergosterol content of dry yeast.	No. of runs.
				gm.	*mg.*	*per cent*	
Zygosaccharomyces mandshuricus...............	2	2	2602	1.4	3	0.2	1
Zygosaccharomyces chevalieri....................	2	2	2605	3.1	6	0.2	1
Nadsonia fulvcscens........	2	4	*	3.5	11	0.3	1
Saccharomyces cerevisiæ...	3	9	*	8.0	16	0.2	1
" " a baking yeast...........	3	9	*	9.6	77	0.8	5
" "	3	9	*	10.4	83	0.8	2
" "	3	9	*	13.9	42	0.3	3
" "	3	9	*	10.0	50	0.5	6
Saccharomyces cerevisiæ, a brewing yeast...........	3	9	*	8.6	120	1.4	2
Saccharomyces carlsbergensis....................	3	9	2345	7.5	150	2.0	3
Saccharomyces pastorianus.	3	9	2339	8.2	25	0.3	3
" *ellipsoideus*.	3	9	*	8.8	53	0.6	4
" *mandshuricus*......................	3	9	2333	1.1	2	0.2	1
Saccharomyces exiguus.....	3	9	2340	2.8	6	0.2	1
" *logos*.......	3	9	2337	1.1	Trace.	Trace.	1
Logos yeast...............	3	9	2703	5.5	39	0.7	4
" "	3	9	†	10.9	98	0.9	3
Saaz "	3	9	2704	7.7	8	0.1	1
Frohberg yeast..........	3	9	2700	6.9	7	0.1	1
" "	3	9	*	8.3	66	0.8	4
Distillers' "	3	9	918	7.5	23	0.3	1
Tokay wine yeast.........	3	9	562	8.9	18	0.2	1
Brewers' yeast	3	9	*	8.4	101	1.2	2
Yeast from malt	3 (?)	9 (?)	‡	9.3	84	0.9	8
" " "	3 (?)	9 (?)	‡	8.1	32	0.4	2
	Non-Saccharomycetaceæ.						
Mycoderma sp............		3	4115	14.2	28	0.2	2
Endomyces lindneri.......		5	2089	5.3	11	0.2	1
" *hordei*.........		5	2088	2.8	6	0.2	1
" *fibuliger*......		5	2090	3.0	6	0.2	1

* Not from the Type Culture Collection.

† A variant of Culture 2703.

‡ Isolated in 1928 from a malt infusion by Dr. Robert P. Myers of this laboratory.

The cultivation was carried on with aeration for 92 hours. The temperature in the laboratory could not be controlled exactly, but thermograph records indicated that the average temperature was 25°.

Before being harvested the cultures were examined microscopically for bacterial contamination, and the occasional contaminated cultures were discarded. The yeasts were harvested by centrifuging, washed in water, and again centrifuged. The volume of sediment was measured, and 5 volumes of 75 per cent methyl alcohol were added. The mixture was shaken vigorously, kept overnight at 13°, and filtered with suction. The filter cake was then placed in a low temperature vacuum oven until it was almost, but not quite dry. The mass was comminuted, passed through a standard 40 mesh sieve, and finally dried in a 75° vacuum oven for 20 minutes. This product constituted the yield of dry yeast per liter of medium reported in Table I.

Certain promising cultures were grown for us on a tonnage basis through the courtesy of Dr. G. S. Bratton of Anheuser-Busch, Inc. We dried these yeasts in large vacuum drum driers, without the use of alcohol, and it is interesting to note that the yields of ergosterol obtained commercially ran parallel with the values calculated for the flask cultures.

Extraction and Determination of Ergosterol.

We found that the method of drying the yeast influences the yield of ergosterol. In order to prepare yeast for efficient extraction it is necessary to dry it rapidly and in such a manner as to give a porous, friable product. The methods of drying described above give a powder from which the ergosterol is readily removed by organic solvents. Preliminary hydrolysis of the yeast cells, recommended by other writers, was found unnecessary with our methods of drying.

Exactly 1 gm. of the dried yeast from each flask culture was extracted by boiling with 20 cc. of alcohol-benzene 2:1 for a few minutes. After the suspended matter settled, the liquid was decanted and filtered. This process was performed five times on each sample, and the fifth time the yeast itself was poured on the filter and washed. The combined filtrates were evaporated to dryness by gently heating under a vacuum. The residue was

dissolved in 95 per cent alcohol, and this solution, suitably diluted, was used to make a series of sector photometer spectrograms. From the observed extinction limits of the 282 mμ ergosterol absorption band, the quantity of ergosterol per gm. of dried yeast was calculated. The above procedure was worked out with the aid of Mr. Warren M. Cox, Jr., of this laboratory.

Spectrographic analysis is probably the most accurate method for estimating small quantities of ergosterol. Its advantage over other methods, colorimetric and gravimetric, lies in its specificity. Ergosterol is not the only chromogenic sterol in yeast, nor the only sterol which is precipitated by digitonin, but in all probability it is the only constituent of fungi which exhibits an ultra-violet absorption band at 282 mμ. The treatment of the cultured yeasts with 75 per cent methyl alcohol removes little, if any, ergosterol, but it is effective in dissolving out the objectionable pigments which exhibit general absorption in the ultra-violet region.

DISCUSSION AND SUMMARY.

Microscopic examination indicated that much of the precipitate produced by autoclaving was redissolved during cultivation; however, the centrifuged yeast showed that some of this sediment remained. Its presence in the yeast caused a slight error in the yield of dry yeast per liter of medium and in the ergosterol content of dry yeast reported in Table I. The former value would be high and the latter correspondingly low.

Of the twenty-nine yeasts investigated, fourteen were cultivated only once, while the fifteen which showed interestingly high ergosterol content were run twice or more. In these repeated runs the average variation in the yield of dry yeast per liter of medium was 13 per cent, and in ergosterol content of dry yeast 19 per cent.

It is evident from Table I that different species of yeast, similarly cultured, may differ enormously in ergosterol content. *Saccharomyces logos* contained but a trace, while *Saccharomyces carlsbergensis* yielded 2 per cent. Different strains of one species, *Saccharomyces cerevisiæ,* ranged from 0.2 to 1.4 per cent. All yeasts of high ergosterol content grew well in culture, but not all which grew well showed high ergosterol content. In fact, the two most

prolific growers, *Mycoderma sp.* and a strain of *Saccharomyces cerevisiæ*, contained a low percentage of ergosterol. In duplicate runs of the same cultures it was observed in thirty-seven out of fifty-three cases that the runs which gave the heavier yield of yeast also showed the higher percentage of ergosterol. It is known that the yield of yeast is markedly influenced by the intensity of the aeration during cultivation.

Heretofore the variations in the ergosterol content of yeast have been attributed largely to differences in the conditions of culture. Our experiments emphasize that the different yeasts exhibit decidedly different capacities for the elaboration of ergosterol, capacities which may be attained or repressed by manipulation of the cultural conditions.

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STUDIES OF GAS AND ELECTROLYTE EQUILIBRIA IN BLOOD.

XVI. THE EVOLUTION OF CARBON DIOXIDE FROM BLOOD AND BUFFER SOLUTIONS.

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Henriques (1928), using Faurholt's data (1924) on the velocity of hydration of CO_2 and the dehydration of H_2CO_3 and HCO_3^- at 0° and 18°, calculated the velocity of dehydration of H_2CO_3 at 38° and pH 7.40. He assumed that under average conditions, in the change from venous to arterial blood in the lung, 11.75 volumes per cent of bound CO_2 must be freed from the erythrocytes. He calculated from Faurholt's data that by change of HCO_3^- and H_2CO_3 into anhydrous CO_2 only 1.95 volumes per cent could be freed. He concluded from this that a large part of the carbon dioxide in the erythrocytes must be in the form of anhydrous CO_2 loosely bound to hemoglobin, "carbhemoglobin." As evidence in confirmation of this hypothesis he showed that during a time when from serum exposed to a vacuum only 22 volumes per cent CO_2 were set free, from whole blood 28 volumes per cent were set free.

Henriques has also explained, by assuming the existence of "carbhemoglobin," the fact that the CO_2 content of the erythrocytes is higher than it would be if the Donnan equation

$$\frac{[HCO_3]_c}{[HCO_3]_s} = \frac{[Cl]_c}{[Cl]_s} = \frac{[H^+]_s}{[H^+]_c}$$

held with activity coefficients of unity for all of the ions concerned. (The subscripts *c* and *s* are used to indicate concentrations in cells and serum respectively.) The fact that the HCO_3 ratio, calculated on the assumption that all the bound CO_2 is in the form

of bicarbonate, is higher than the Cl ratio was shown by Warburg (1922) and by Van Slyke, Hastings, Murray, and Sendroy (1925). The latter authors demonstrated that the HCO_3 ratio thus calculated is, in terms of molar concentrations, 25 per cent higher than the Cl ratio, and assumed that the difference was due to the effect of unknown factors on the activity coefficients of the two types of ions. Both the Cl and the HCO_3 ratios were found higher than the ratios of H^+ activities determined by the hydrogen electrode. Henriques stretches his theory to explain also the fact that the Cl ratio is higher than the H^+ activity ratio; he assumes that not only part of the CO_2, but a part, somewhat less, of the Cl in the cells is also bound to the hemoglobin.

Regardless of the mechanism attributed to it and the hypotheses based on it, the behavior of hemoglobin in accelerating the evolution of CO_2 from blood is a phenomenon of sufficient physiological importance to deserve confirmation.

Henriques' experimental data do not appear in themselves sufficient to demonstrate such an acceleration. He showed that when whole blood was agitated in an evacuated chamber CO_2 escaped from the fluid more rapidly than from serum similarly treated. Such a difference could, in part at least, be attributed to the well known acidic and buffer properties of hemoglobin, which retard the rise of pH when CO_2 escapes from whole blood, and thereby make it possible completely to free the latter of CO_2 by agitation in an evacuated space; while serum exposed to a vacuum becomes so alkaline (from the change $NaHCO_3 \rightarrow Na_2CO_3$) that only about half of the CO_2 can be removed. For comparison with blood, it appears essential to use a solution similar, not only in initial CO_2 content and pH, but also in buffer value, so that throughout the evolution of CO_2 the pH of the compared solution shall approximate that of whole blood or hemoglobin solution which has lost similar proportions of its CO_2.

We have therefore prepared solutions of bicarbonate, carbonic acid, and phosphates which meet these requirements. When agitated under reduced pressure they do, in fact, evolve CO_2 at a much slower rate than whole blood or solutions of hemoglobin. The results thoroughly confirm Henriques' conclusion that hemoglobin accelerates the escape of CO_2 gas.

That the acceleration is due to the existence of quantitatively

important proportions of CO_2 bound in the form of "carbhemoglobin," however, appears to us improbable for reasons that will be given in the discussion of the results.

EXPERIMENTAL.

Equilibration of Blood, Serum, and Solutions with Carbon Dioxide.

The blood serum or solutions were saturated at 38° with 40 mm. of CO_2, the double tonometers described by Austin *et al.* (1922) being used. 100 cc. of solution were saturated with 1000 cc. of gas. The lower chamber of the tonometer was filled with solution and clamped off from the upper except during the period of saturation. After 1 hour's saturation, the gas mixture was renewed twice and two $\frac{1}{2}$ hour saturations were made in order to make certain that the final conditions should approximate as closely as possible those desired.

The CO_2 tensions were determined, as described in connection with the saturation method, Austin *et al.* (1922), by analysis, by Haldane's method, of the separated gases in the upper chamber of the tonometer.

The total CO_2 contents of the solutions were determined by means of the manometric apparatus of Van Slyke and Neill (1924), the factors of Van Slyke and Sendroy (1927) being used to calculate the amount of CO_2.

The hemoglobin concentration was determined by the colorimetric method of Palmer (1918).

The amount of protein in the serum was determined by the gasometric micro-Kjeldahl method of Van Slyke (1927).

Symbols Used.

The following symbols are used:

$[BHCO_3]$, $[H_2CO_3]$, $[CO_2]$ represent concentration in mM per liter of $NaHCO_3$, physically dissolved CO_2, and total CO_2 respectively in the solutions.

β represents the molecular buffer value for the buffers in solution other than sodium bicarbonate.

The carbon dioxide tensions in mm. of mercury are expressed as p_{CO_2}.

α is the Bunsen solubility coefficient of CO_2.

(Hb) represents concentration of hemoglobin in mM per kilo of blood.

Constants.

The values of pK′, used in the equation $\text{pH} = \text{pK}' + \log \frac{[\text{BHCO}_3]}{[\text{H}_2\text{CO}_3]}$ were 6.15, 6.10, and 6.17 for blood, serum, and 0.08 M phosphate solution, respectively. The value of pK′ for whole blood was taken from the nomogram of Van Slyke, Hastings, Murray, and Sendroy (1925). The value of pK′ for serum was taken from Hastings, Sendroy, and Van Slyke (1928). The value of pK′ for bicarbonate in the phosphate mixtures was calculated from the equation $\text{pK}' = 6.33 - 0.5\sqrt{C}$, where C, the calculated concentration in mols per liter, is substituted for μ, the ionic strength used by Hastings and Sendroy (1925) for bicarbonate and sodium chloride mixtures, when the effect of the phosphates, mol for mol, is assumed to be the same as that of sodium chloride and bicarbonate.

The solubility coefficient for CO_2, α_{CO_2}, was 0.485, 0.511, and 0.528 for blood, serum, and 0.08 M phosphates, respectively (Van Slyke, Sendroy, Hastings, and Neill (1928)). The value of α_{CO_2} for the phosphate mixtures was obtained by subtracting the appropriate value for the decrease in solubility of CO_2 due to the concentration of the phosphate and bicarbonate; *i.e.*, $0.08 \times 0.2 = 0.016$ and $0.02 \times 0.13 = 0.002$ from $\alpha_{CO_2} = 0.546$ for water.

The value of pK′ = 6.79 for the phosphate solution used in the equation $C = \frac{\beta}{0.575U}$ was taken from Cohn (1927).

Calculations.

The concentration of $[H_2CO_3]$ was estimated from the formula $[H_2CO_3] = 0.591\ \alpha_{CO_2} \times p_{CO_2}$. The concentration of $[BHCO_3]$ was determined by subtracting $[H_2CO_3]$ from $[CO_2]$. The calculation of pH from $[BHCO_3]$ and $[H_2CO_3]$ was made by use of the Henderson-Hasselbalch equation (Van Slyke, Sendroy, Hastings, and Neill (1928)).

The buffer value of serum was calculated as $0.104 \times$ gm. of

serum protein per liter, the albumin:globulin ratio being assumed to be 1.6 (Van Slyke, Hastings, Hiller, and Sendroy (1928)).

The concentration of hemoglobin necessary to give a desired buffer value was calculated by means of Equations 52 and 17 (Van Slyke, Wu, and McLean (1923)), $\beta = 3.6\ (\text{Hb}) + 0.068\ (\text{P})_s$, where $(\text{P})_s = 0.072 - 0.0039\ (\text{Hb})$.

The buffer value of blood was calculated from the hemoglobin content by the above equation.

The concentration in mols per liter of phosphate of given pH required to give a desired buffer value was calculated as $C = \frac{\beta}{0.575U}$ by the use of Fig. 9 in the paper of Van Slyke on buffers (1922). The U curve there shows, for a pH point at any given distance either side of the pK′ of the given buffer, the percentage of maximal buffer value that will be exerted at that pH. The maximum buffer value of any buffer solution is 0.575 C and is exerted when pH = pK′, C being the molar concentration of the buffer, in this case phosphate. Hence the buffer value of any phosphate is 0.575 CU, where U is the factor from Fig. 9.

Evolution of CO_2 from Blood, Serum, and Buffer Solutions under Reduced Pressure at Various Time Intervals.

A solution with the required pH and double the concentration of phosphate necessary to give the calculated buffer value of blood, and a solution with double the required amount of sodium bicarbonate necessary to give the same $[NaHCO_3] : [H_2CO_3]$ ratio at 40 mm. tension of CO_2 as blood were made up. The two solutions were then mixed, giving a solution with the same pH, buffer value, and $[NaHCO_3] : [H_2CO_3]$ ratio as that of blood.

Undiluted ox blood and the above buffer solution were used. The red cells of the blood were laked with 0.1 per cent saponin. Formation of lactic acid was prevented by the addition of 0.1 per cent sodium fluoride. The saponin and sodium fluoride were added to the phosphate solution as well as to the blood so that conditions in both solutions would be the same. The blood and phosphate solutions were then equilibrated at 38° with CO_2 at 40 mm. tension of mercury.

The amount of CO_2 evolved at various time intervals from blood and buffer solutions was determined by means of the Harington-Van Slyke constant volume apparatus (1924).

The apparatus was washed once with N H_2SO_4 and then three times with distilled water to insure that it was free of acid. The apparatus was evacuated and shaken. The mercury was then run back into the apparatus and any air expelled through the upper stop-cock. 1 drop of octyl alcohol was introduced into the apparatus. The upper stop-cock was sealed with mercury and the apparatus evacuated till the mercury was just above the lower stop-cock. The lower stop-cock was then closed.

The blood or solution was then transferred quantitatively by means of the 5 cc. pipette, described by Van Slyke and Neill in Section XII of their paper (1924). One arm of the Y-shaped stop-cock at the top of the pipette was joined by means of a short rubber tube to the side arm on the lower stop-cock of the Harington-Van Slyke extraction chamber. Mercury was expelled from the lower chamber of the Harington-Van Slyke apparatus through the Y arm of the Ostwald pipette to remove any air. The Y stop-cock was turned and the solution was run into the lower bulb of the gas apparatus, followed by mercury to seal the stop-cock of the gas apparatus. Care was taken that the blood or solution was run into the apparatus without being placed under reduced pressure at any time. The pipette was then detached from the gas apparatus.

The solution was allowed to run into the upper chamber of the gas apparatus along with sufficient mercury so that the meniscus of the mercury was at the 50 cc. mark. The apparatus was at once shaken vigorously for the required time interval. The solution was then drawn down into the lower chamber, leaving the gas in the upper chamber. The time required to admit the solution to the upper chamber of the Harington-Van Slyke apparatus and drain it out was 10 seconds.

The solution was expelled from the lower chamber of the apparatus through the side arm on the lower stop-cock along with enough mercury to seal the stop-cock.

5 cc. of acidified distilled water were admitted to the upper chamber from the cup at the top and shaken in the evacuated chamber to establish equilibrium with the CO_2 there. The lower stop-cock of the extraction chamber was opened and mercury was admitted until the water meniscus was exactly at the 2.0 cc. mark; then a manometer reading, p_1, was taken.

TABLE I.

Evolution of CO_2 from Ox Blood and Solutions with Same Buffer Value, pH, CO_2 Tension, and CO_2 Content as That of Blood.

Solution.	pCO_2	$[H_2CO_3]$	Total $[CO_2]$.	$[BHCO_3]$	$\frac{[BHCO_3]}{[H_2CO_3]}$	pH	Hb concentration in terms of O_2 capacity.	β	Temperature.	CO_2 evolved during various time intervals. Time of shaking, sec. mM per liter. 5	10	20	40	80	Per cent of total CO_2. 5	10	20	40	80
	mm.	*mM*	*mM*	*mM*			*vol. per cent*		*°C.*										
Ox blood.	41.0	1.19	20.68	19.49	16.4	7.37	20.1	30.71	23.5	3.87	3.96	4.18	4.39	4.44	19.98	20.28	21.40	22.48	22.73
	41.5	1.20	19.54	18.35	15.4	7.34	21.7	33.04	23.0	3.26	3.39	3.73	4.02	4.38	16.70	17.36	19.10	20.58	22.42
	41.6	1.20	22.28	21.08	17.6	7.39	20.0	30.58	23.0	3.12	3.50	4.16	4.40	4.38	15.40	15.70	18.90	19.75	19.67
Buffer 0.08 M phosphate and bicarbonate.	40.0	1.25	20.38	19.13	15.3	7.36		30.59	24.0	1.69	1.80	2.16	2.78	3.89	8.29	8.83	10.61	13.65	19.11
	40.6	1.26	19.98	18.72	14.9	7.34		30.59	23.5	1.64	1.78	2.10	2.74	3.73	8.20	8.91	10.51	13.72	18.66
Buffer 0.08 M phosphate, bicarbonate, and ox blood.	40.0	1.23	20.85	19.62	16.0	7.37	1.1	30.59	25.0	4.72	5.36	5.69	6.12	6.26	22.62	25.70	27.30	29.35	30.00
	41.2	1.28	20.80	19.52	15.3	7.35	1.0	30.59	24.0	4.92	5.39	5.59	6.10	6.26	23.66	25.91	26.88	29.32	30.10

3 drops of 5 N NaOH were introduced through the upper stop-cock to absorb the CO_2. After the stop-cock was again sealed with mercury, the meniscus of the water was brought to the 2.0 cc. mark and a second reading, p_2, taken.

The CO_2 evolved from the blood or solution was then determined by multiplying $p_2 - p_1$ by the factors calculated from the formula of Van Slyke and Sendroy (1927) with the constants $a = 2.0$, $S = 5.0$, and sample = 5.0 cc.

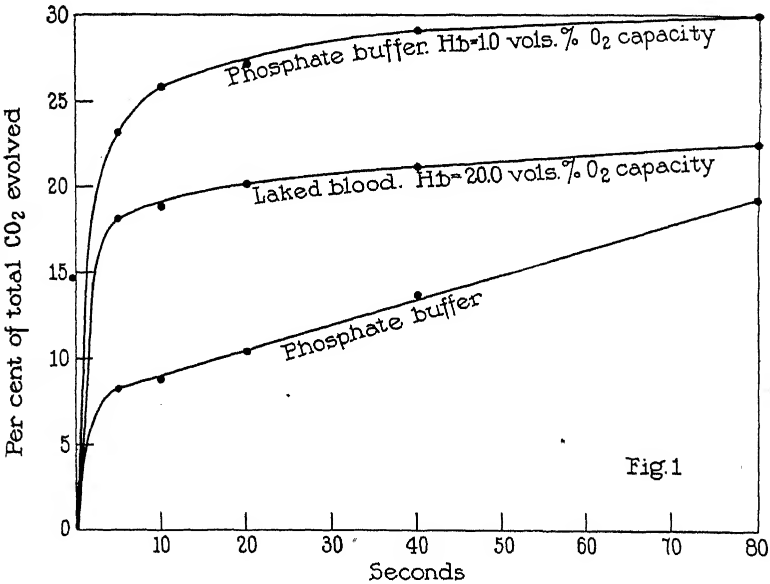

FIG. 1. Comparison of the rate of liberation of CO_2 from whole ox blood, phosphate buffers with the same buffer value as that of blood, and from similar phosphate buffer containing red blood cells. Ordinates represent per cent of total CO_2 liberated; abscissæ represent seconds of shaking in the Harington-Van Slyke apparatus.

The results of these experiments are shown in Table I and Fig. 1. It is seen that as much CO_2 is evolved from the blood in 10 seconds as from the phosphate solution in 80 seconds. The evolution of CO_2 from the phosphate solution proceeds at a steady ratio after the first 5 seconds, 40 per cent of the total CO_2 being liberated during the first 5 seconds. The evolution of CO_2 from laked blood

TABLE II.

Evolution of CO_2 from Ox Serum and Solutions with Same Buffer Value, pH, CO_2 Tension, and CO_2 Content as That of Serum.

Solution.	pCO_2	$[H_2CO_3]$	Total $[CO_2]$.	$[BHCO_3]$	$\frac{[BHCO_3]}{[H_2CO_3]}$	pH	Hb concentration in terms of O_2 capacity.	β	Temperature.	CO_2 evolved during various time intervals. Time of shaking, sec. 5	10	20	40	80	5	10	20	40	80
										mM per liter.					Per cent of total CO_2.				
	mm.	*mM*	*mM*	*mM*			*vol. per cent*		*°C.*										
Ox serum.	42.0	1.27	26.20	24.93	19.7	7.39		7.78	23.0	1.26	1.36	1.78	2.32	2.89	4.81	5.19	6.79	8.86	11.04
	41.3	1.25	25.51	24.26	19.4	7.38		7.90	25.0	1.34	1.47	1.93	2.50	3.13	5.30	5.76	7.56	9.80	12.27
Ox blood laked, 0.1 M sodium chloride, and bicarbonate.	42.0		24.62				4.8	7.34	23.5	3.00	3.23	3.54	3.55	3.57	11.98	13.15	14.43	14.46	14.54
	43.0		27.04				4.7	7.20	23.0	2.94	3.17	3.76	3.96	3.98	10.88	11.74	13.92	14.67	14.72
Ox serum.	38.7	1.17	27.58	26.41	22.6	7.45	0.3	6.98	24.0	2.11	2.50	2.77	3.25	3.42	7.66	9.06	10.05	11.78	12.40
	41.0	1.24	30.25	29.01	23.4	7.47	0.3	7.08	25.0	2.15	2.34	2.72	3.44	3.73	7.11	7.74	9.00	11.37	12.33
Ox blood unlaked, 0.1 M sodium chloride and bicarbonate.	42.0		24.62				4.8	7.34	23.5	1.92	2.24	2.69	3.11	3.36	7.82	9.12	10.96	12.66	13.68
	43.3		24.71				4.7	7.20	23.0	1.99	2.53	2.83	3.24	3.58	8.03	10.24	11.44	13.12	14.48

is very rapid during the first 5 seconds as 80 per cent of the CO_2 freed is liberated during this interval. This agrees with the finding of Henriques ((1928) p. 5) that CO_2 is evolved more rapidly when hemoglobin is present in solution.

In order to determine whether a solution free of hemoglobin, but containing other proteins in sufficient concentration to give a similar buffer value, would behave differently, similar experiments were performed with undiluted ox serum.

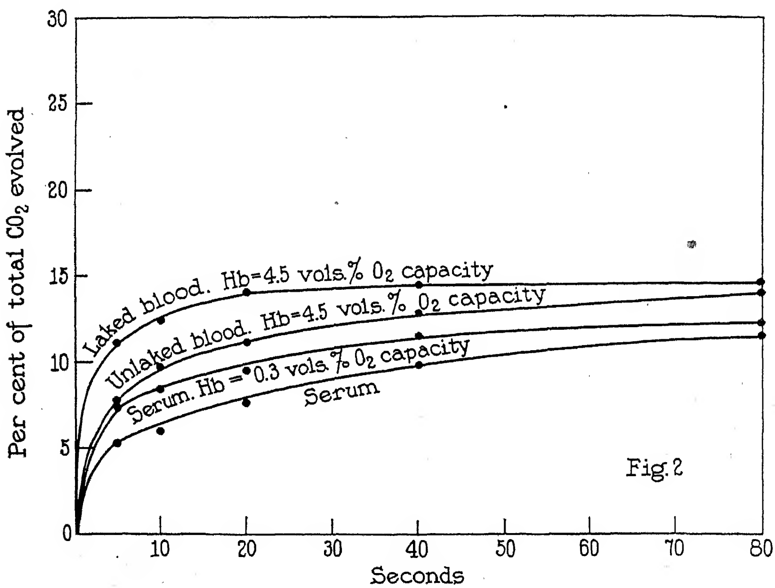

FIG. 2. Comparison of the rate of liberation of CO_2 from serum and from blood diluted so that its buffer value is the same as that of serum. Ordinates represent per cent of total CO_2 liberated. Abscissæ represent seconds of shaking in the Harington-Van Slyke apparatus.

Undiluted ox serum and ox blood, diluted with solution containing 100 mM of sodium chloride and 25 mM of sodium bicarbonate to give the same buffer value as that of serum, were used. The red cells of the blood were laked with 0.1 per cent saponin. Formation of lactic acid was prevented by the addition of 0.1 per cent sodium fluoride. The saponin and sodium fluoride were added to serum as well as to the blood solution so that conditions would

be the same in both. In a second series of experiments the saponin was not added so that the red cells were unlaked. The serum and blood mixtures were then equilibrated at 38° with CO_2 at 40 mm. tension of mercury.

The results of these experiments are shown in Table II and Fig. 2. It is seen that as much CO_2 is evolved from the diluted laked blood solution in 5 seconds as from serum in 80 seconds.

The evolution of CO_2 from serum proceeds at a steady rate between 5 and 40 seconds and then falls off slowly. The evolution of CO_2 from the diluted blood with the same buffer value and at the same tension as the serum is very rapid during the first 5 seconds; 73 per cent of the CO_2 liberated is freed during this interval. After 20 seconds shaking, no more CO_2 escapes from this solution.

This finding again agrees with those of Henriques in attributing to hemoglobin a unique ability to accelerate the evolution of CO_2 from its solution.

The diluted unlaked blood, the serum of which contained 0.3 per cent hemoglobin, and the serum containing 0.3 per cent hemoglobin gave curves which lay between those of the laked blood solution and serum.

To ascertain the relation between hemoglobin concentration and the accelerating effect of hemoglobin on CO_2 evolution, a relatively slight proportion of red cells was added to the bicarbonate-phosphate buffer solution previously described.

6 cc. of ox blood were added to 120 cc. of the buffer of 0.08 M phosphate and 0.02 M bicarbonate containing 0.1 per cent saponin and sodium fluoride to make the hemoglobin concentration about 5 per cent that of whole blood. The mixture was saturated at 38° with CO_2 at 40 mm. tension. Portions of the solution were then agitated in the evacuated Harington-Van Slyke chamber, and the amounts of CO_2 evolved in various time intervals were determined.

It is seen from Table I and Fig. 1 that the addition to the buffer solution of 0.05 the concentration of red cells that exists in blood increased the rate of liberation of CO_2 from solution so that it was comparable with the rate of liberation of CO_2 from the whole blood. A larger amount of CO_2 was evolved from the blood-buffer solution than from the blood, but the ratio of initial evolution speed to amount of gas finally liberated was approximately

the same, 77 per cent of the finally liberated amount of CO_2 escaping in the first 5 seconds.

DISCUSSION.

Henriques' conclusion, that the presence of hemoglobin accelerates the evolution of CO_2 from blood, is entirely confirmed by the above results.

However, his hypothesis that the acceleration is due to the binding of a great part of the CO_2 in the cells in the form, not of bicarbonate, but of anhydrous CO_2 gas loosely united to hemoglobin, does not appear to be consistent with our finding that 0.05 the concentration of hemoglobin present in blood causes, in a solution of similar pH and buffer power, an approximately equal acceleration of CO_2 evolution.

Furthermore, Henriques' assumption that an important proportion of the bound CO_2 is not in the form of bicarbonate is inconsistent with quantitative data published in previous papers of this series, the significance of which Henriques appears to have overlooked. The amounts of alkali bound by hemoglobin at varying pH levels have been determined independently by two methods. (1) Crystals of purified hemoglobin have been dissolved in known amounts of standard NaOH or KOH solution, which was then saturated with CO_2 at known tensions, the quantitative relationships of hemoglobin, alkali, pH, and CO_2 being so chosen that they resembled those occurring in blood *in vivo* (Van Slyke, Hastings, Heidelberger, and Neill (1922); Hastings, Van Slyke, Neill, Heidelberger, and Harington (1924)). It was assumed in calculating the results of these experiments that the alkali not bound to hemoglobin was combined with CO_2 in the form of bicarbonate. The base, BHb, bound to hemoglobin was therefore calculated as follows:

$$BHb = (\text{total alkali added}) - BHCO_3$$

the bicarbonate being calculated as

$$BHCO_3 = (\text{total } CO_2) - H_2CO_3$$

The symbol, H_2CO_3, is used to represent all the free dissolved carbonic acid, both that actually in the form of H_2CO_3 and in the

form of anhydrous CO_2. The amount of such free carbonic acid was calculated from the determined tension of CO_2 gas and the solubility coefficient of CO_2 in water solutions of the nature employed.

It is obvious from the formulæ of calculation that if important amounts of CO_2, in excess of those calculated from the solubility of the gas in water solutions, had remained in a form other than bicarbonate, a gross error would have been caused in the calculated value of $BHCO_3$, and consequently of BHb.

(2) However, Hastings, Sendroy, Murray, and Heidelberger (1924) found the same proportions of alkali combining with hemoglobin at the same pH levels by an entirely different procedure, carried out without CO_2. They dissolved hemoglobin in measured amounts of NaOH solution and titrated back with HCl, determining the pH after each HCl addition by means of Hastings' rotating hydrogen electrode. For reduced hemoglobin and for carbon monoxide hemoglobin (which has the same alkali-binding power as oxyhemoglobin) the same proportions of alkali bound per mol of hemoglobin at the same pH were found, within the limits of experimental error, as by the CO_2 titration method, outlined above.

It therefore appears that, if the accuracy of these results is accepted, one must either (1) accept the assumption that even in the presence of hemoglobin practically all of the CO_2 is divided between the forms of free carbonic acid and bicarbonate, or else (2) one must assume that, even on the alkaline side of the isoelectric point, free HCl forms with hemoglobin the same sort of indefinite compound that Henriques has assumed CO_2 to form, and in the same proportions at the same pH levels. The latter assumption is, we believe, too far outside the limits of probability to merit consideration. The fact that titration of alkali hemoglobinate with HCl and with CO_2 both indicate the same alkali-combining power of hemoglobin constitutes, in the belief of the writers, evidence against the existence of quantitatively important proportions of CO_2 in any form such as that assumed for "carbhemoglobin" by Henriques.

SUMMARY.

The rates of evolution of CO_2 from blood and phosphate buffers have been determined. As much CO_2 is liberated from blood or erythrocyte solution in 5 seconds as from a phosphate or serum protein solution with the same CO_2 content and buffer value in 80 seconds. These findings agree with those of Henriques.

The addition of red cells to the phosphate buffer in sufficient quantity so that the hemoglobin concentration is 0.05 that in blood accelerates the liberation of CO_2 so that the rate is as rapid as from whole blood.

We have at present no explanation of the mechanism by which the cell contents (probably but not certainly hemoglobin) accelerate CO_2 evolution from solution.

The direct combination of anhydrous CO_2 with hemoglobin assumed by Henriques is without proof of existence. That the carbon dioxide in the blood is accounted for by the amounts present as bicarbonate and physically dissolved carbon dioxide has, furthermore, been shown by quantitative data published from this laboratory.

The rapidity of evolution of CO_2 from evacuated blood solutions appears to be best described as due to a catalytic acceleration by the cell contents of the reaction $HCO_3^- + H^+ \rightleftharpoons H_2CO_3$. Concerning the mechanism of the catalysis no data are at present available for the formation of a reasonably probable hypothesis.

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THE PREPARATION OF LIPOID-FREE SERUM.

APPARATUS FOR EXTRACTION AT LOW TEMPERATURES.

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In connection with some work on the concentration of pneumococcus antibody, it appeared to be desirable to prepare considerable quantities of lipoid-free serum. The methods available (1–3) called for precipitation with alcohol or with acetone in the cold, filtration, and extraction with ether. The technique described by Hewitt (3) was first employed. It gave excellent results with 100 cc. of serum. However, when a liter of serum was used, two large Buchner funnels were required and, in the absence of a refrigerator room, it was difficult to keep these filters sufficiently cold. Other objections were the large amounts of ether vapor that escaped into the laboratory and the cost of the absolute alcohol and anhydrous ether.

Other methods were devised. During the course of the work, it was discovered that some of the original observations that had suggested the preparation of lipoid-free serum were probably erroneous. Therefore, none of the methods developed has been employed on the large scale originally contemplated nor will the work be continued. However, the apparatus used and the results obtained appear to be of interest and may be of value to others.

An attempt was made to substitute for the acetone or alcohol, used by previous investigators, other organic liquids that are miscible with both water and ether and that might be used at room temperatures. Ethylene glycol was found to resemble glycerol in not precipitating the serum proteins. Precipitates were obtained with mesityl oxide, 1,4-dioxane, and diacetone alcohol; but these, when filtered out, were insoluble in water or salt solution. Evidently, coagulation had occurred. These liquids, therefore, offered no advantage over alcohol or acetone.

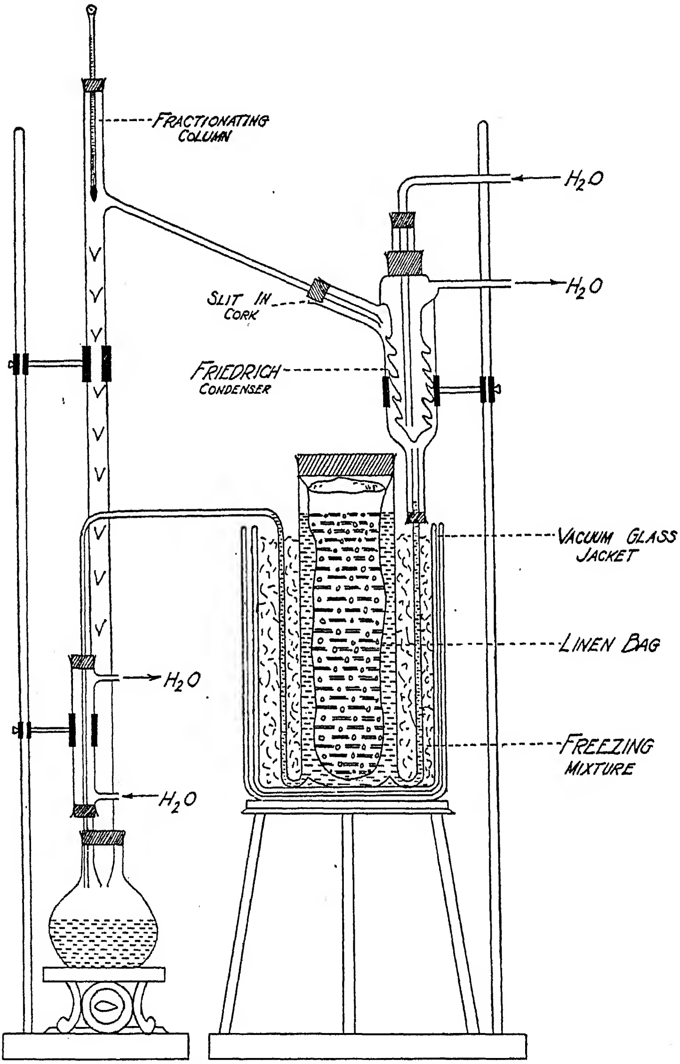

FIG. 1. Continuous extraction apparatus for solids.

It was then determined to utilize the low temperatures obtainable by the use of solid carbon dioxide, now commercially available. In the first experiment, the serum was dropped into ether kept at $-50°$. The frozen drops of serum were then transferred to a

continuous extraction apparatus similar to the one shown in Fig. 1. The serum was kept frozen by immersion of this part of the apparatus in a suitable bath. Granular calcium chloride was added to the flask containing the boiling ether. It was found that the frozen serum tenaciously retained both its water and its lipoid, even though the temperature of the bath was raised to $-10°$.

In other experiments, the serum, at a temperature of 5°, was run into 6 or 7 volumes of synthetic methyl alcohol, kept at $-50°$, with constant stirring. The mixture was then filtered through a bag of fine linen cloth in the apparatus shown in Fig. 1. The bag was suspended from the edge of the apparatus by three bent wires. This part of the apparatus was kept cold by a bath of alcohol or kerosene and solid carbon dioxide. As soon as the temperature of the vapor at the top of the column reached 67°, the flask was changed for one containing fresh methyl alcohol. When water was no longer being extracted in appreciable quantities, the flask containing methyl alcohol was replaced by one containing ether previously dried over $CaCl_2$. After three or four extractions, this flask was replaced by one containing fresh ether and anhydrous calcium chloride. The extraction was continued while the temperature of the bath was allowed to rise to 15°. As the temperature rose, the calcium chloride liquefied, indicating that water or alcohol was being given off to the ether. The extraction flask was then replaced by one containing fresh granular calcium chloride. Finally, this remained granular.[1]

The linen bag was then removed from the apparatus, whirled rapidly in the air for a few minutes, and then dried over sulfuric acid in a vacuum desiccator. The material thus obtained was snow-white and powdered readily.

Four preparations with methyl alcohol and one with ethyl alcohol were obtained. The latter and one of those with methyl alcohol were promptly treated with water. They redissolved completely to give clear solutions. The other three preparations were kept, in the dry state, over the summer. These did not redissolve completely. However, when tested, all five prepara-

[1]The same retention of alcohol, or water, by the precipitated protein was observed by Hewitt, who used metallic sodium to remove it from the system as rapidly as it was extracted by the ether.

tions were found to have retained their original content of protective antibody.

The solutions of the dried and extracted sera were analyzed for lipoid phosphorus by the method of Fiske and Subbarow (4) and for fatty acids and cholesterol by those of Bloor, Pelkan, and Allen (5). Large amounts of the alcohol-ether filtrates were required in order to secure satisfactory readings. The results obtained are shown in Table I.

TABLE I.

Lipoid Content of Serum 99, before and after Extraction.

Results are given in mg. per 100 cc.

	Original.	Ethyl alcohol and ether.	Methyl alcohol and ether. Preparation No.				N-Amyl alcohol.	Diethyl carbinol.	Isoamyl alcohol.
			1	2	3	4			
Lipoid phosphorus	5.1	<0.5	0.5	0.6	0.53		0.8	<0.5	0.3
Cholesterol	82.9	<2	6.8	3.3	4.6	5.0	15	5.5	2.1
Fatty acids	73	30	20	4	19	23	11	42	34

TABLE II.

Lipoid Content of Serum 220, before and after Extraction with Isoamyl Alcohol.

Results are given in mg. per 100 cc.

	Original.	After extraction.
Lipoid phosphorus	4.8	0.5
Cholesterol	47.6	3.6
Fatty acids	178	2.4

This method, while interesting, was scarcely applicable to the desired purpose because only small quantities of serum could be treated at a time. It was decided to try extraction with immiscible solvents. For this purpose, the ordinary types of extraction apparatus were modified to secure thorough cooling of the extracting liquid before it was allowed to pass through the serum.

The apparatus used with chloroform, carbon disulfide, and carbon tetrachloride resembled that shown in Fig. 2, except that the condensed solvent was introduced at the top and drawn off from the bottom, the interrupted syphon previously employed by

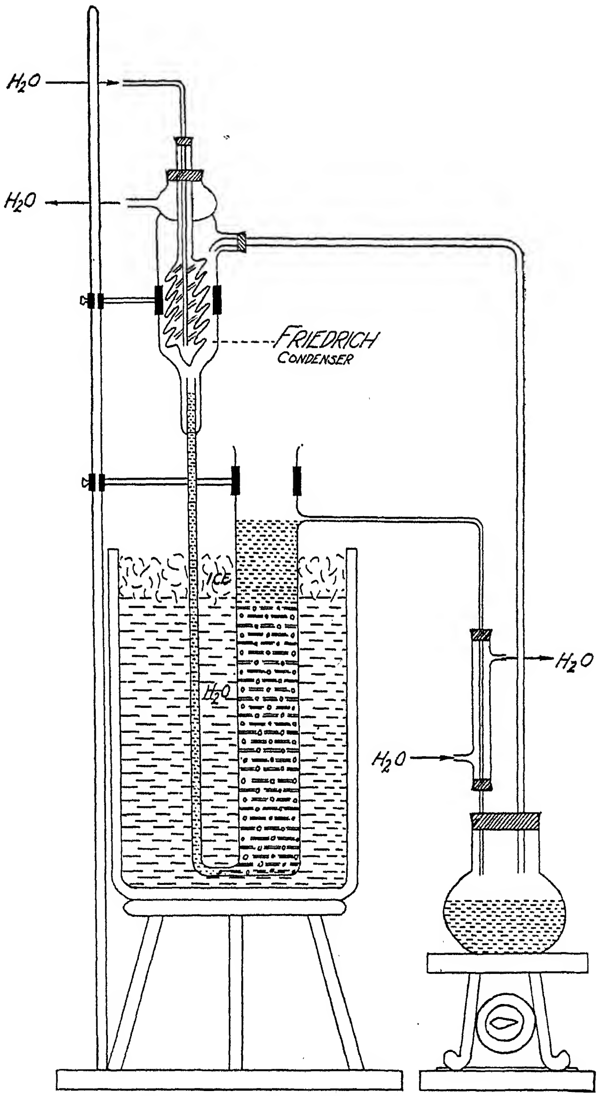

Fig. 2. Apparatus for extraction with immiscible solvents.

one of us (6) being used. Analyses showed that no appreciable quantity of lipoid phosphorus was extracted by any of these solvents.

Similar results were obtained with ether and ligroin, with the apparatus shown in Fig. 2. However, the various amyl alcohols were effective. As is shown by the figures in Tables I and II, normal amyl alcohol, diethyl carbinol ($(C_2H_5)_2CHOH$), and isoamyl alcohol ($CH_3(C_2H_5)CHCH_2OH$) removed all except traces of cholesterol and lecithin. Tertiary amyl alcohol, $(CH_3)_2C_2H_5COH$ could not be used because of its marked coagulating action.

It is interesting to observe the rather high values for fatty acids in Serum 99 after extraction with diethyl carbinol or with isoamyl alcohol. Their significance is not at all clear but they suggest the possible presence of larger amounts of non-extractable soaps in Serum 99 than in Serum 220. However, since no analyses for soap content were made, some other factor may have been responsible.

During the extraction with the amyl alcohols, there was considerable coagulation of protein. However, this was not accompanied by any decrease in content of protective antibody that could be detected by the usual mouse method.

It was of interest to ascertain whether or not the extraction of the phospholipids was due to the coagulation of the protein component of a peculiar lipid-protein compound. Accordingly some of Serum 220 that had been extracted with isoamyl alcohol was left in contact with lecithin for several days and was then filtered. Analysis showed it to contain 2.2 mg. of lipoid phosphorus per 100 cc. It was then extracted with ether in a continuous extraction apparatus. After thorough extraction, analysis showed it to contain 2.1 mg. of lipoid phosphorus per 100 cc. Apparently, the previous treatment of the serum with isoamyl alcohol had not affected the ability of the serum to retain lecithin in a form not extractable by ether. This experiment would seem to indicate that protein-lipid compounds of the kind postulated by Macheboeuf (7) may be formed by simple mixture of serum and lecithin.

It is a pleasure to express our thanks to Miss Georgia Cooper and Miss Helen M. Dedrick of the Research Laboratories of the Department of Health of New York City, for the testing of the antibody content of our preparations and to Mr. Raymond Miro for the drawings accompanying this paper.

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ON THE FATE OF DIIODOTYROSINE IN THE ANIMAL ORGANISM.*

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Recent discoveries in the chemistry of the thyroid gland have revived interest in the chemical and physiological properties of diiodotyrosine. Harington and Randall (1) isolated this substance from normal hog thyroids, and Foster (2) found that it constituted over 30 per cent of the total iodine of thyroglobulin. The possibility, therefore, that diiodotyrosine may be an intermediate in the formation of thyroxine (Harington and Barger (3)) or that it may play a rôle in normal and abnormal thyroid function makes desirable a study of its behavior. The present paper reports the results of an investigation of the fate of this substance in the animal organism. The subject has been investigated previously by others, most notably by Oswald (4).

Oswald fed 10 gm. of diiodotyrosine to a rabbit over a period of several days and searched for iodine compounds in the excreta. The total iodine of the substance fed was well recovered in the urine and feces, about 95 per cent being in the urine. Of the total iodine in the urine about 46 per cent was present as inorganic iodide; the rest was still in organic form. Fractionation of the urine yielded unchanged diiodotyrosine, and a crystalline acid melting at 75° which contained 48 per cent iodine and 2.1 per cent nitrogen. It seems probable that this unidentified substance was an impure specimen of the new compound which is described in the present report.

In the work here reported rabbits were the experimental animals. They were fed an ordinary diet of oats and cabbage. The urine

* This work was aided by the Research Grant from the Chemical Foundation to the Department of Biological Chemistry.

and feces were collected separately. Diiodotyrosine mixed dry with one-fourth its weight of sodium bicarbonate was administered by mouth in gelatin capsules. The experiment was begun with six rabbits but two of the animals died during the experimental period.[1] The spontaneously voided urines were collected daily at the same hour, and were pooled together. An aliquot from each day's output was analyzed for total and for inorganic iodine. The rest was saved for the isolation work.

Total iodine was determined by a slightly modified Kendall (5) method. The modifications consisted of the use of nickel dishes heated over free flame instead of crucibles in the furnace described by Kendall, filtration of the alkaline solution of the melt through an asbestos mat in a Gooch crucible, the use of a few drops of phenol to remove the last of the excess bromine after the greater part had been driven off by a few minutes boiling, and acidification with sulfuric instead of phosphoric acid.

Inorganic iodide was determined as follows: 10 cc. of urine were mixed with 1 cc. of 50 per cent sulfuric acid in a centrifuge tube. After standing a few minutes the small, dark colored, flocculent precipitate was centrifuged down. Of the clear supernatant fluid 10 cc. were placed in another centrifuge tube and mixed with 5 cc. of saturated silver sulfate. After standing 10 minutes in the dark the silver halides were centrifuged down, the supernatant fluid decanted, and the precipitate washed twice in the centrifuge tube with approximately 0.1 N sulfuric acid. After the wash water was decanted, 3 cc. of saturated bromine water were added and the precipitate ground and stirred with a glass rod for 4 to 5 minutes, thereby oxidizing the silver iodide to iodate. The mixture was then diluted to about 12 to 14 cc. with water, the silver halides centrifuged down, the supernatant fluid was decanted to a small flask, the tube and precipitate were washed with water, and the washings added to the flask. Then the solution was boiled till most of the bromine was expelled, cooled, treated with 2 or 3

[1] One rabbit died of pneumonia, the other by an accident in feeding the capsules. Mild toxic symptoms in the form of anorexia and loss of weight appeared after a few days. Autopsies at the conclusion of the experiment showed gastroenteritis with injection of the vessels of the whole intestinal tract. The thyroids were slightly enlarged and hyperemic, and two on microscopic examination were found to be very rich in colloid. The kidneys were normal.

drops of phenol to remove the last of the bromine, and the iodate titrated with potassium iodide and thiosulfate. This procedure has been checked against known potassium iodide solutions and found to give satisfactory results, both in the presence and absence of added diiodotyrosine.

Table I gives an abstract of the daily progress of the experiment. A total of 68 gm. of pure recrystallized diiodotyrosine (40 gm. iodine) was fed. Of this, 33 gm. of iodine were found in the urine and 4.1 gm. in the feces for the period, or a total recovery of 93 per cent. A point of interest to be seen in Table I is the remarkably constant ratio of inorganic iodide to total iodine in the urine. The remainder of the urine samples (approximately 12 liters) was mixed and worked up in lots of 2 or 3 liters in the following manner.

TABLE I.

Iodine in Urine after Feeding Diiodotyrosine.

Date.	Iodine in substance fed.	Urine.				No. of rabbits used.
		Volume.	Total I_2.	Inorganic I_2.	$\frac{\text{Inorganic } I_2.}{\text{Total } I_2.}$	
1929	*gm.*	*cc.*	*gm.*	*gm.*	*per cent*	
Oct. 17	1.92	980	0.93	0.07	7.5	6
" 18	3.84	900	2.34	0.23	9.8	6
" 19	3.84	775	2.86	0.31	10.8	6
" 20	3.84	1100	3.35	0.36	10.7	6
" 21	0	1150	2.00	0.21	10.5	6
" 22	3.19	1090	2.40	0.25	10.4	5
" 23	3.19	720	2.42	0.23	9.5	5
" 24	2.55	470	1.31	0.13	9.9	4
" 25	2.55	840	2.44	0.26	10.6	4
" 26	2.55	710	2.00	0.22	11.0	4
" 27 } " 28 }	2.55 0	1440	3.25	0.32	9.9	4
" 29	1.92	825	1.43	0.17	11.9	4
" 30	1.92	790	1.57	0.23	14.6	4
" 31	3.84	790	2.22	0.25	11.4	4
Nov. 1	1.28	535	1.34	0.14	10.5	4
" 2	1.28	435	0.69	0.10	14.5	4
" 3 } " 4 }	0 0	715	0.45	0.10	22.2	4

Each lot was strongly acidified to Congo paper with sulfuric

acid, and filtered from a small amount of flocculent precipitate which contained little or no iodine. It was then extracted with ether which had been shaken with acidified potassium iodide and with sodium bisulfite to destroy peroxide. The ether extract, which contains about one-third of the total organic iodine, was distilled, leaving a brown tarry residue. This residue was repeatedly extracted with small amounts of boiling water. The aqueous extract, on cooling, yielded long needle crystals which increased when acidified with hydrochloric acid. Recrystallized from hot water, the substance melted at 73–75° then as the temperature was raised, resolidified and again melted rather sharply at 158–159°. About 70 per cent of the ether-soluble organic iodine was recovered as apparently pure crystals of this compound. The urine, after ether extraction, still strongly acid with sulfuric acid, was shaken with butyl alcohol. All the remaining organic iodine was thus transferred to the butyl alcohol with remarkable rapidity. The butyl alcohol was distilled off under reduced pressure and the residue recrystallized from hot 50 per cent acetic acid and yielded, in the best case, 90 per cent of the organic iodine in this fraction as pure diiodotyrosine, m.p. 204°. No evidence for other organic iodine compounds could be obtained. The organic iodine unaccounted for (20 per cent of the total organic iodine in the best case) could easily be explained as due to losses in isolation of the compounds mentioned above, namely, unchanged diiodotyrosine and the ether-soluble compound.

The ether-soluble compound appears to be a substance hitherto undescribed, namely, the α-hydroxy acid corresponding to diiodotyrosine, for the following reasons.

1. It contained no nitrogen.

2. It gave no derivative with semicarbazide or *p*-nitrophenylhydrazine.

3. 7.39 mg. and 9.42 mg. required 2.075 and 2.680 cc. of 0.098 N thiosulfate.

5.267 mg. substance: 4.80 mg. CO_2 and 0.85 mg. H_2O.

$C_9H_8O_4I_2$.	Calculated.	C 24.88,	H 1.86,	I 58.7.
	Found.	" 24.86,	" 1.81,	" 58.5.

4. One would expect the acidity of the phenolic group in such a compound to be increased so that it should be almost completely

neutralized when titrated with alkali to the phenolphthalein end-point. The substance should, therefore, titrate like a dibasic acid of equivalent weight approximately 434 ÷ 2 = 217. 7.39 mg. and 9.42 mg. required 1.20 and 1.55 cc. of 0.0275 N $Ba(OH)_2$. Hence the neutral equivalent = 224, 221.

5. When deiodinated by hydrogenation with palladium calcium carbonate catalyst of Busch and Stöve (6) the substance used 2 mols of hydrogen. 0.4860 gm. of the substance required 56.8 cc. of hydrogen at 23° and 758 mm. The blank was 1.3 cc.

$C_9H_8O_4I_2$. Calculated. 50.1 cc.
Found. 51 cc. at 0° and 760 mm.

6. The iodine-free substance was recovered from the reaction mixture by extraction with ether and when recrystallized from benzene formed very fine needles which melted not very sharply at 141–143°.

5.094 mg. substance (dried at 110°): 11.09 mg. CO_2 and 2.56 mg. H_2O.

$C_9H_{10}O_4$.	Calculated.	C 59.31, H 5.54.
	Found.	" 59.37, " 5.62.

The iodine-containing acid was feebly dextrorotatory as the free acid but became more strongly dextrorotatory when converted to its sodium salt.

For 69.3 mg. in 2 cc. the observed rotation in a 1 dm. tube = +0.11°. When neutralized with sodium carbonate, the observed rotation = +0.23°.

Hence according to Levene's rule (7) we are dealing with a substance spatially related to *l*-lactic acid. But, that the substance is present as nearly the racemic mixture, is indicated by the fact that the *p*-oxyphenyllactic acid resulting on deiodination showed a specific rotation of only −2.8° whereas Kotake (8) has reported −18.05° for the levo form of this acid. It is not likely that racemization occurred during deiodination with the palladium catalyst since Harington (9) has shown that it does not occur when *l*-diiodotyrosine is similarly deiodinated.

The authors gratefully acknowledge the assistance received from their colleague, Dr. O. Wintersteiner, who carried out the micro combustion analyses.

SUMMARY.

After diiodotyrosine was fed to rabbits approximately 10 per cent of the total iodine in the urine was recovered as inorganic iodide, 60 per cent as unchanged diiodotyrosine, 18 per cent as 3,5-diiodo-4-hydroxyphenyllactic acid, and 12 per cent could not be isolated.

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CHEMICAL STUDIES OF MUSCLE CONTRACTURE.

IV. CHANGES IN PHOSPHORUS, NITROGEN, AND FAT PRODUCED BY TETANUS TOXIN.

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This work was undertaken for the purpose of determining what changes might occur in acid-soluble phosphorus in mammalian muscle in contracture produced by tetanus toxin[1] and what relation they might bear to contractures in general. By comparing them with changes seen in frog muscle in contracture produced by a different agent (1) we wished to see whether the phosphocreatine would be reduced and whether the relationship between phosphorus and protein nitrogen would be maintained as it was in the frog muscles. Fat was also determined to see what changes might accompany contracture.

Rabbits were chosen on account of their resistance to the toxin and the ease of producing local tetanus in one limb without noticeable involvement of the opposite limb. As in the previous study of glycogen changes (2), the toxin was injected subcutaneously into the popliteal space of one hind limb. When contracture in the gastrocnemius had developed (6 to 10 days after the injection of toxin) the animal was anesthetized by a combination of amytal and spinal block. The two gastrocnemii were exposed, dissected free from surrounding structures, and frozen *in situ* with a mixture of carbon dioxide snow and ether. They were prepared for the determination of acid-soluble phosphorus according to the procedure previously given (3). Inorganic

[1] The toxin used was gratuitously furnished by Parke, Davis and Company.

phosphate was determined by precipitation as the calcium salt according to Fiske and Subbarow (4). Other fractions were determined as in Paper III of the series (5). Nitrogen values represent essentially protein nitrogen and were made by standard Kjeldahl procedure on the muscle residues after the extraction with 5 per cent aqueous trichloroacetic acid. Without the protein nitrogen value, as previously demonstrated (1), and also as

TABLE I.

Distribution of Acid-Soluble Phosphorus in Gastrocnemii of Rabbits with Local Tetanus in One Hind Limb.

Muscle No.	Days after injection.	Acid-soluble P,* mg. per 100 gm.					N	Ratio total P:N.
		I	Pc	Py	R	TP		
							mg. per gm.	
1 T	8	21	63	36	35	155		
1 C		22	76	43	34	175		
2 T	8	19	70	41	35	165	30.2	5.5
2 C		21	81	45	34	182	32.2	5.7
3 T	6	19	71	48	34	172	30.2	5.7
3 C		20	80	54	38	192	33.0	5.8
4 T	10	23	54	47	46	170	28.1	6 0
4 C		16	81	56	31	184	31.4	5.9
5 T	9	21	59	41	51	172	28.7	6.0
5 C		18	84	45	41	188	31.5	6.0

T = tetanus; C = control; N = protein nitrogen.

* I = inorganic; Pc = phosphocreatine; Py = second acid-labile fraction; R = relatively acid-stable remainder; TP = total phosphorus.

found by other workers (6), it was not possible to judge whether decrease in total phosphorus was absolute or relative.

Table I shows that there was no constant change in inorganic phosphate when tetanus and control muscles were compared. Phosphocreatine was always slightly less in the tetanus muscle, even after correction for increased water content, but the tetanus muscles were also more irritable and the twitching which occurred in them during freezing probably lowered the phosphocreatine.

The decrease in phosphocreatine in these muscles was neither so constant nor so marked as it was in frog muscle, for in two of the animals (Animals 2 and 3) the difference is well within the limits of variation of the method. The total phosphorus was always less in the tetanus muscle, but it will be seen in Table I that when the ratio of total phosphorus to nitrogen was computed, the reduction in phosphorus paralleled the reduction in protein nitrogen; hence the essential change appeared to be one of water content. The increased water content of the tetanus muscle was opposite to the change in water content found to predispose frog muscle to contracture (1), for the latter had been dehydrated by injections of hypertonic solutions.

TABLE II.

Comparison of Quantities of Total Fatty Extractives Obtained from Tetanus and Control Gastrocnemii.

Fat is expressed in mg. per gm.

Rabbit No.	6	7	8	9	10
Tetanus	17.7	20.2	23.0	20.0	18.9
Control	17.3	20.4	20.7	18.8	16.6

This study as well as the previous study on frog muscle serves to emphasize that the acid-soluble phosphorus behaves as if it were as much an integral part of the structure of muscle as the protein nitrogen.

Fat Content.

A second series of five rabbits was prepared by injections of tetanus toxin for determination of fat content of the muscles. Muscle samples were taken 9 days after injection, in a manner similar to the first, except that the animals were killed by a blow and the muscle samples cut into thin slices without freezing. The samples were extracted with acetone at room temperature for 4 to 6 weeks, then dried, ground fine, and extracted in a Soxhlet apparatus with ether. The acetone extract was evaporated to dryness and the chloroform-soluble part of the residue combined with that extracted by ether. The total fatty residue was dried at 55° and weighed. The results are given in Table II. A higher value for the tetanus muscle was seen in four of the five pairs but the increase in Rabbit 6 was within the limits of error of the

method. Other workers have found that the amount of fat is not changed in muscle under various experimental conditions of short duration. Cuthbertson's (7) experiments showed no change in fat content of fatigued muscle. Winfield (8) likewise found no marked change in total fat content of muscle in fatigue, rigor in oxygen and in hydrogen, and in recovery from fatigue. Audova (9), however, found that fat accumulates in muscles undergoing atrophy as a result of severance of the nerve and following section of the tendon. The accumulation of fat was slow at first and more rapid in the later stages, while fat-free dry substance diminished rapidly at first and at a slower rate in later stages, indicating that fat increase is probably not at the cost of disintegrated protein. The duration of our experiments was too short for atrophy to have progressed beyond initial stages but the small increase in fat found indicates agreement with Audova's findings.

SUMMARY.

The changes in acid-soluble phosphorus found during tetanus contracture consisted of a reduction in the total phosphorus (proportional to the protein nitrogen), a decrease in phosphocreatine which was somewhat greater than would be accounted for by water change in the muscle, and an inconstant variation of other fractions.

The fat content was increased 10 per cent in the tetanus muscle in three and essentially unchanged in two animals. It therefore seems to have no significance for the acute stage of contracture.

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FAT METABOLISM.

I. A STUDY OF THE RATE OF DIGESTION OF FATS AS DETERMINED BY THE CHYLOMICRONS OF THE BLOOD.

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In 1924, Gage and Fish (1) published a method for the determination of fat digestion, absorption, and assimilation by the dark field microscope and a fat-soluble dye, Sudan III. By means of the dark field microscope, the visible lipemia of the blood, due to small fat particles, called chylomicrons, can be studied during the digestion of a fat by the use of 1 drop of blood each hour. To gain further evidence on the rate of digestion of fats with this method, the present investigation was undertaken.

The fats used in this study were butter from cow's milk, butter from the milk of goats, cod liver oil, butter and bran, and butter and cellophane, to determine with one subject the difference in the rate of digestion of different fats, and with different subjects the rate of digestion of fat with roughage.

The subjects for the experiments on which the present study is based were college women in good physical condition, and healthy white rats. The fats to be tested were generally fed with carbohydrate food. After a fasting period of 12 to 15 hours, the test meal was eaten. A drop of blood was taken every hour during the digestive cycle for 7 or 8 hours, and was examined with the dark field microscope. Samples of blood from the central and the peripheral circulations of the rat were first compared. No differences in the chylomicron count were found, consequently blood for the experiments with rats was taken from the end of the tail and blood from humans was taken from the tip of a finger.

Curves were plotted with the chylomicron count as ordinate and the hours at which the counts were made as abscissa. Com-

parisons were made also of the areas enclosed by the curves by using the trapezoidal rule and finding the mean ordinates, which method is said to be accurate for straight line figures.

$$\frac{\frac{\text{First} + \text{last}}{2} + \text{intermediate}}{n - 1}$$

where n = the number of cases. This was done to compare with Gage's method of finding the areas enclosed by the curves. According to Gage, not only the height of the curve but the area would indicate approximately the rate and time of the process of adding fat to the blood during a digestive cycle. Gage used the planimeter for finding the area, but also, he weighed the irregular

TABLE I.

Areas, Found by the Trapezoidal Rule, Enclosed by the Curves from the Chylomicron Counts of Human Subjects.

Test meal.	Areas.*
20 gm. butter from cow's milk	39.2
17 " cod liver oil	36.9
20 " butter from goat milk	33.0
30 " fat and bran	31.0
Fat-free meal (boiled rice and jam)	15.5

* The figures represent the means of the ordinates in Charts I and II.

areas drawn on two-ply drawing paper and compared this with a known weight of a square centimeter of the same paper found by weighing 250 square centimeters of the paper. The present writer, after using the planimeter and comparing the results with those obtained by the rule used in calculus, concluded that the latter method was as accurate as the former, hence areas in this paper are based on the system of mean ordinates (Table I).

After a fasting period of 12 to 15 hours, the subjects came to the laboratory at 8 a.m. A sample of blood was taken and the subjects were fed the test meal, consisting of the fat to be tested, 20 or 30 gm. as shown later in this paper; two slices of bread, weighing approximately 40 gm., and 75 cc. of orange juice. Every hour thereafter a drop of blood was taken until 3 or 4 o'clock in the afternoon. At noon a fat-free meal was fed. In this meal

were approximately 125 gm. of boiled rice, 90 gm. of strawberry jam, salted crackers weighing approximately 25 gm., and apple sauce, 200 gm., plus 35 gm. of sugar. The apples used for the

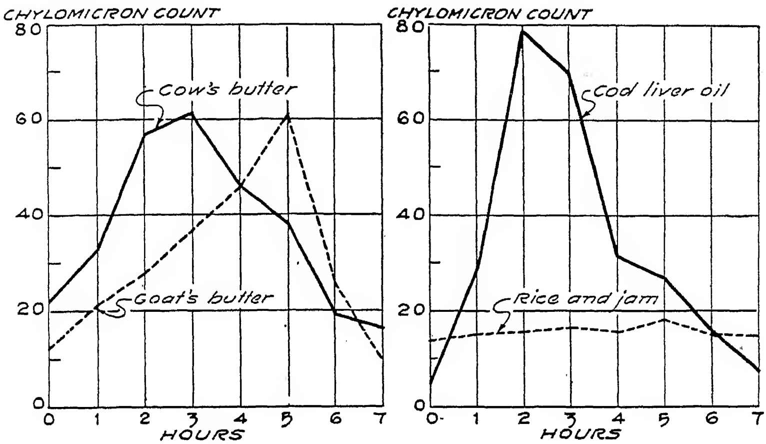

CHART I. Relative rapidity of digestion of butter from cow's milk, butter from goat milk, cod liver oil, and boiled rice and jam, based on the chylomicron counts of human subjects. The amount of butter from cow's milk and of cod liver oil digested in the first 2 or 3 hours is much greater than that of butter from goat milk. The data for the above chart are based on three cases each. The averages of the three cases on butter from cow's milk agree with the averages of 88 cases reported by Gage and Fish (1). A record sheet was kept for each individual: her age, height, weight, diet for the preceding 24 hours, the test meal fed, the fat-free meal at noon, and the chylomicrons in the blood at each hour in five fields on the slide. Though only three individuals are represented in the averages above, they are the same three subjects and the chylomicron counts of each of the three were similar, for example, when butter from goat milk was fed, the peak of the curve occurred at the 5th hour for each subject, and at that time the number of chylomicrons found in the blood of one of the subjects was 65, in a second subject, 65.4, and in the third, 52.8. The butter from goat milk used in these experiments was made in the laboratory. The milk was pasteurized, separated at the dairy, and made into butter by shaking in a Mason jar. It was salted and used on toast as was the butter from cow's milk.

apple sauce were about 145 gm. before cooking. In the experiments where bran was fed, 20 gm. was the allowance for each subject. During the experiments, the subjects were allowed to

drink water in any quantity desired. Throughout the day the subjects continued their ordinary work, attending classes, studying, and walking short distances.

The results of feeding 20 gm. of butter from cow's milk compared with feeding 20 gm. of butter from goat milk indicate, from the chylomicron count, that although the digestion of butter from goat milk is almost as complete as that of butter from cow's milk, the rate of digestion is much slower (Chart I). The peak of the curve for the rate of digestion as measured by the chylomicron count, occurs at the end of 3 hours when the butter from cow's milk is fed and at the end of 5 hours when butter from goat milk is fed. It has long been thought that goat milk is in some way superior to cow's milk for infant feeding. This may be based mainly on the results of empirical observations for the data found in the literature (2) do not wholly verify such a statement.

Results similar to the above were found on feeding butter to white rats. Practically the same results were found when cornstarch was fed to white rats. Accordingly, carbohydrate in the form of boiled rice and jam was fed to humans to determine the shape of the digestion curve, as measured by the chylomicrons in the blood after a fat-free meal, and to compare it with the fat digestion curves already discussed. This carbohydrate digestion curve, although not a perfectly straight line is a very flat curve indicating little increase in fat particles with the length of the digestion period (Chart I). The very small rise and fall may be due merely to the intake of the carbohydrate food. McClure and Huntsinger (3) found that the mg. of fatty acids and the mg. of cholesterol per 100 cc. of blood, increase after the ingestion of fat, of protein, and of a fat-free meal. They suggest the increase is due to the mobilization of lipids from the body tissues.

Cod liver oil was also fed to humans. 17 gm. of cod liver oil which are comparable in fat content to 20 gm. of butter, approximately 82 per cent fat, were used in the experiment (Chart I). The cod liver oil curve indicates rapid utilization of the fat. The peak of digestion appears at the 2nd hour as compared with the 3rd hour for butter from cow's milk. The higher peak and steeper slope indicate that the cod liver oil is absorbed more rapidly by the blood than is butter either from cow's milk or goat milk. Work of Wells (4), who fed 100 gm. of cod liver oil per day to human sub-

jects, indicates that it was well assimilated—the coefficient of digestibility was 97. Judged by these results (4), cod liver oil slightly increased the thoroughness of digestion of other fats present in the experimental ration.

After obtaining results with fats alone, it was proposed to find out, with the chylomicron count, how the feeding of bran with fats affected the digestion curve. Accordingly, 30 gm. of fat with 20 gm. of bran were fed at the first meal of the experimental day (Chart II). For eighteen of the thirty humans on this fat and bran diet, the peak of the curve for the rate of digestion occurred

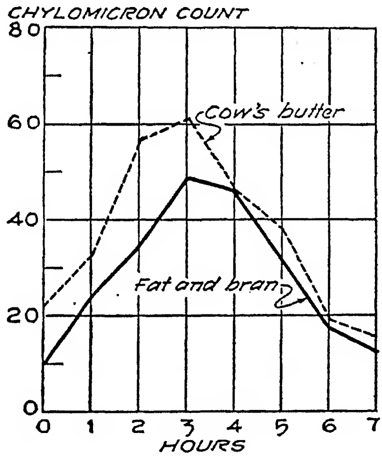

CHART II. Relative rapidity of digestion of fat and bran, and butter from cow's milk, based on the chylomicron counts of human subjects. The data for the above chart are based on thirty cases on fat with bran. The curve for butter from cow's milk is the same as in Chart I.

after 3 hours; for eleven after 4 hours; and for one after 5 hours. Eight of the thirty young women were overweight and three were underweight. The peaks of the curves of seven of the overweight individuals occurred after 3 hours. The peaks of the curves of two of the underweight subjects occurred after 4 hours and that of the third, a very much underweight individual, occurred after 3 hours. This would indicate that no definite conclusions can be drawn about the utilization of fat in over- and underweight individuals from this chylomicron count. The average results of the

thirty cases of feeding 30 gm. of fat with 20 gm. of bran indicate slower digestion than when 20 gm. of butter are fed without bran, under the conditions of these experiments. The probable errors for the averages for each hour of the digestive cycle varied from 10 ± 0.8 at the zero hour to 48.8 ± 2.8 at the 3rd hour, at which time the greatest number of chylomicrons were found. The coefficient of variation, obtained by the formula, $\frac{\text{standard deviation}}{\text{mean}} \times 100$, was

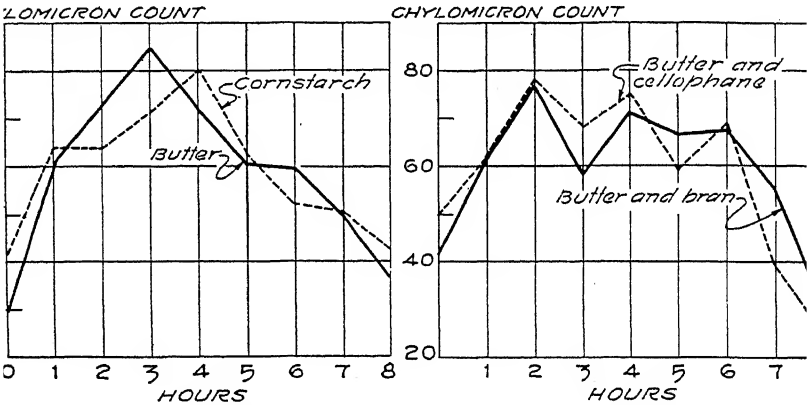

CHART III. Relative rapidity of digestion of butter, corn-starch, butter and bran, and butter and cellophane, based on the chylomicron counts of white rats. These data are based on five cases each, three males and two females on each diet. A litter of eight rats for example, was so divided that there were two animals, a male and a female of approximately the same weight, on each of the four test diets. The conditions in the laboratory were the same for all the litters. The rats were without food for 15 hours preceding the experiments and in individual cages with raised bottoms.

46 at the 3rd hour and 67 at the zero hour. This probably indicates that there is less variation in the chylomicron count at the 3rd hour than at the zero hour where the number of chylomicrons present in the blood after fasting for 12 to 15 hours varies greatly.

At about the same time that these experiments were in progress, similar comparisons were made with white rats. 6 weeks old rats were selected and well-matched for the four test diets used, as follows: 1 gm. of butter alone, 20 gm. of corn-starch alone, 1 gm.

of butter and 1 gm. of bran, and 1 gm. of butter and ½ gm. of cellophane. 1 gm. was found to be the maximum amount of fat that all the animals would eat immediately, after fasting 15 hours. The corn-starch was before the rats for 4 hours, for it was found that in 1 hour, even after fasting overnight, the animals would rarely eat a weighable amount. Of the 20 gm. fed, the amounts eaten varied from 4 to 7 gm. Cellophane, which is almost pure cellulose, was used in order to make a comparison with bran as a source of roughage. It was found by the chylomicron count that the digestion curves for all four diets differed little (Chart III). Since these results were obtained with white rats, it was thought advisable to study in greater detail the fat metabolism of the rat before continuing experiments with this animal, with use of the dark field microscope.

Work is now in progress in this laboratory to accumulate more data on humans and to compare the amount of fat found in the blood by means of the chylomicron method, with the lipids in the blood by chemical analysis.

SUMMARY.

The experiments described deal with the rate of digestion of fats alone and fats with roughage as determined by means of the dark field microscope, by a study of the small fat particles, called chylomicrons, of the blood.

The subjects on which the studies were based were healthy white rats, and college women in good physical condition, who were without food for 12 to 15 hours preceding the experimental period.

Digestion curves, based on the chylomicron counts are critically considered.

Under the conditions of the experiments, butter from cow's milk and cod liver oil are taken up by the blood much faster than are the same amounts of either butter from goat milk, or butter from cow's milk when eaten with bran.

Grateful acknowledgment is made of the suggestions of Dr. Adelaide Spohn, and of helpful advice on the method given by Dr. S. H. Gage, when this work was undertaken.

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A SIMPLE TEST FOR LEVULOSE (KETOSE?) IN GLUCIDES.*

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INTRODUCTION.

During an attempt to obtain a permanent pink color for a method of estimating bile salts in the blood by the Pettenkofer reaction (1), one of us observed that levulose and other carbohydrates that contain the levulose molecule behaved quite differently from other carbohydrates, in respect to both the delicacy and the speed of the reaction. It thus appeared that a reverse Pettenkofer test might be applied to a method of detection of levulose in much the same manner as is done in the Molisch test, with the bile salts instead of α-naphthol.

The method finally devised gives a clear demarcation between the levulose reaction and that of other carbohydrates in ordinary conditions, and may be useful, particularly when only a small amount of the sample is available for a test.

Method.

In a small test-tube (preferably 1 cm. in diameter) place 1 cc. of 0.1 per cent fresh aqueous solution of the ordinary ox bile salts. Then add 1 cc. of concentrated H_2SO_4, shake immediately, and quickly add to the mixture a drop of a solution to be tested. An immediate production (say in about 15 seconds) of pink or purple color on the top of the solution indicates the probable presence of levulose, free or in combination.

As a class exercise to show what sugars give the test, it is convenient to take a drop from each of several 0.1 M solutions of various carbohydrates, an ordinary 6 inch test-tube being used, but the following precautions should be emphasized.

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The bile salts must be conjugated and their aqueous solution must be made fresh. A permanent stock solution can be kept in alcohol, but its solvent must be evaporated off before the desired concentration can be prepared in water.

Since the temperature is a very important factor in this reaction, the sample drop must be added quickly after shaking which must be done immediately after the addition of the acid to the salts. Under the condition described above the temperature of the mixture just before the addition of the sample drop is found to be about 85°. It should be remembered that among the factors that influence the temperature of this reaction medium are such as size and thickness of the test-tube, order and speed of mixing the acid with the bile salts, and size and number of the sample drops.

EXPERIMENTAL.

As many makes of carbohydrates as were obtainable in the market were used, including the C.P. special (Pfanstiehl) brands. Their solutions were prepared in such a way that a known volume, not weight, contains a definite amount of sugar. The minimum concentration that gives a positive test was determined by dilution of these solutions, a single drop always being used for the test. The single drop from the pipette we used was a twenty-seventh of 1 cc. at the room temperature, as measured by distilled water. Exactly the same sized test-tubes (1 cm. in diameter) were used in all comparative experiments, and readings of the results were made at 15 seconds after the introduction of the drop.

The results are as follows:

The minimum concentrations of the carbohydrates containing levulose that give a positive test are 0.03 per cent for levulose, 0.03 per cent for inulin, 0.06 per cent for saccharose, 0.09 per cent for melezitose, and 0.1 per cent for raffinose. If we ignore the amount of water eliminated in the condensation of the hexose to form these higher carbohydrates—which is roughly 5 per cent for each molecule of the hexoses—our results would indicate that this test quantitatively detects levulose, if the drop contains roughly 0.00001 gm. or more levulose in free or combined form; that is, the concentration must be at least about M/600 or more. A slight exception in the case of raffinose might be due to a comparatively large amount of water of crystallization that this sugar is known to contain.

These results seem to show that the levulose molecule alone is responsible for the reaction and that other aldose hexoses present in the above carbohydrates do not interfere with the test. The results with other carbohydrates show, however, that all the sugars give the test if the concentration is high enough. Thus glucose gives it at 10 per cent, maltose 8 per cent, glycogen 8 per cent, starch 8 per cent, and dextrin 2.5 per cent. Free glucose is, therefore, 300 or more times less sensitive than levulose. We are almost tempted to interpret this fact as due to a possible equilibrium that glucose may maintain with levulose in an aqueous solution. There is, however, no reason to suppose this reaction to be specific for levulose alone. But, regardless of the reason why concentrated glucose will give this test, a useful fact remains in that the difference between the minimum concentration required for glucose, as compared with that of levulose, is so great that one will have no difficulty in distinguishing them by this test. As a matter of fact, almost a single dilution will decide the point.

Among other sugars, galactose gives the test at 4 per cent concentration, lactose at 8 per cent, and mannose at 3 per cent. The color produced by a 2 per cent solution of arabinose and 4 per cent gum arabic is orange instead of pink or purple.

DISCUSSION.

All these results being considered together, it appears that this test is not absolutely specific for levulose, and we have no reason to doubt that other ketone sugars which we have not tested and which are known to give the ordinary levulose tests, such as sorbose, will also give positive results with this reaction. Within the concentrations we described, and the possible presence of such compounds as furfural, which of course gives this reaction, being eliminated, with this test one can single out the presence of levulose and probably other ketone sugars (?).

A preliminary application of this test was made with a few substances. The undiluted plasma of the blood collected before breakfast gives no levulose reaction even if taken from a severely hyperglycemic patient. The plasma of a normal person, whose blood before the ingestion of 150 gm. of saccharose was negative, gave a strongly positive reaction 1 hour after, even when diluted with an equal amount of distilled water, so that we roughly estimate

the presence of at least 0.06 per cent levulose in the blood either free or partly in the form of saccharose.

No samples of human or cow's milk ever gave a positive test. This is not surprizing in view of the fact that even in human milk the concentration of lactose present is below the amount necessary to give the test.

Normal urine will almost always give this levulose reaction. If the color be entirely due to levulose in the urine, it means that normal urine contains about 0.06 per cent levulose. This amount does not seem possible in the light of the recent report of Van Slyke and Hawkins (2) who find that normal urine does not ordinarily contain more than 0.02 per cent fermentable sugars. Whatever the nature of the compound that is responsible for this reaction found in urine, an interesting fact remains that the normal plasma does not contain enough to give the test, while the urine does.

The hexosephosphate prepared in this laboratory by Dr. Foulger failed to give this test.

It is interesting to note that a sample of thymonucleic acid prepared in this laboratory, but the purity of which has not yet been ascertained by analysis, was found to give this test in a one-half of 1 per cent solution. From this it seems that not all, if any, of the carbohydrate group in the nucleic acid can be an ordinary aldose. It is interesting in this connection to recall the suggestion of Steudel and Peiser (3) that thymonucleic acid may contain the levulose nucleus. However, the recent work of Levene and London (4) is believed by them to show that thymonucleic acid contains not a hexose but a deoxidized aldehyde pentose sugar of the general nature of a desoxy-*d*-ribose, which later was identified definitely as 2-desoxy-*d*-ribose by Levene, Mikeska, and Mori (5). If this proves to be the sugar really present in thymonucleic acid, it would indicate that this class of sugar gives this reaction, but in a concentration about 10 times that necessary for levulose.

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LIPID STUDIES IN XANTHOMA.

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Studies of the chemical composition of cutaneous xanthoma have resulted in the interesting finding that the lipids in such tissues contain considerable amounts of cholesterol. According to some investigators the occurrence of these tumors is associated with a hypercholesterolemia, whereas in the opinion of others this is not the case. Our experience with this question has been that while the cholesterol content of the blood of some individuals having these tumors is high, the amount of the sterol in the blood of others falls within the normal limits. It would appear therefore that the parallelism described by some investigators is not essential and that a hypercholesterolemia is merely incidental and not a requisite for the occurrence of the tumors. A report of our previous studies has already been made (1, 2).

The present communication is a continuation of our studies of the chemical nature of the lipids in xanthoma. An unusually large pedunculated tumor weighing 45 gm. was removed from the buttock in a case diagnosed as typical xanthoma tuberosum. Macroscopically the tumor mass, about the size of a small hen's egg, was on cross-section seen to be composed of a uniform soft saffron-colored material apparently well encapsulated. Surrounding it was what appeared to be normal subcutaneous fat. This subcutaneous fatty connective tissue was of normal color and sharply differentiated from the tumor. It was possible to make a clean cut separation of the two types of tissue. An opportunity, therefore, presented itself not only for making a more detailed analysis of the lipids in xanthoma, but also for comparing this analysis with that made on the adjacent normal subcutaneous fat.

The total lipids were secured from the tissues by extracting them first with hot absolute alcohol, then with anhydrous ethyl ether,

and finally with chloroform. The combined extracts were then evaporated *in vacuo* to a small volume and the lipids removed from the concentrate by extraction with petroleum ether (b.p. 20–40°).

In Table I are summarized some of the results obtained in this investigation. Added to that summation are the values previously secured by one of us (3) for the fatty substances of the normal subcutaneous fat of man. It is evident from our results that the lipids in the tissue directly in contact with the tumor are much like those in the normal subcutaneous fat of man. On the other hand, the lipids in the tumor are undoubtedly of another type.

TABLE I.

Comparison of Nature of Total Lipids of Xanthomatous Tumor with Those Present in Tissue Surrounding the Tumor and in Normal Human Subcutaneous Fat.

		Iodine No.		Phospholipids.		Cholesterol.		Arachidonic acid, total fatty acids.	Linoleic acid, total fatty acids.
	Total lipids.	Total lipids.	Total fatty acids.	Total lipids.	Tissue.	Total lipids.	Tissue.		
	per cent			*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Xanthomatous tumor.......	17.6	80	106	8.10	1.42	48.81	8.59	0.90	3.40
Tissue surrounding tumor...	35.1	64	71	0.46	0.16	0.97	0.34	0.36	0.67
Normal human subcutaneous fat..................		65		0.04		0.24		0.33	0.50

The iodine number of the fatty substances obtained from the tumor is considerably higher than that found for the other lipids, being 88 as compared with 64 for the lipids surrounding the tumor and 65 for normal human subcutaneous fat. This suggests at once the presence of more highly unsaturated fatty acid in the abnormal fat. The latter is, however, very highly pigmented and inasmuch as subsequent examination of the pigment fraction made it apparent that carotene, an unsaturated hydrocarbon, is the principal pigment in the tumor it might be possible that the difference in the iodine numbers of the lipids can be ascribed to a variation in pigment content. That this is not the case is evident

from a further consideration of the data in Table I, where it is shown that the iodine number of the total fatty acids secured from the tumor fat is 106 as compared with 71, the similar constant obtained for the fatty acids isolated after the saponification of the total lipids in the adhering fat. This difference is even more marked than the one existing between the iodine numbers of the original fatty substances and is a more exact index of the degree of unsaturation of the fatty acids contained in the fats, since the non-saponifiable matter and almost all of the pigment were removed from the total fatty acid fractions. A further study of these fractions was made by employing the well known bromination method for the determination of the more highly unsaturated fatty acids. As a result of this it was found (Table I) that while 0.9 per cent of the fatty acids in the abnormal tissue is in the form of arachidonic and 3.4 per cent as linoleic acid, only 0.36 per cent of arachidonic and 0.57 per cent of linoleic are found in the total lipids of the adhering fat. One of us (3) had previously reported 0.33 per cent of arachidonic and 0.50 per cent of linoleic acid present in normal subcutaneous fat. This finding was soon confirmed by Wagner (4). It is thus very clear that so far as iodine number and content of the more highly unsaturated fatty acids are concerned, the adhering fat is like normal human subcutaneous fat and that the tumor fat is very different.

Table I also shows that the tumor fat differs from the others not only with regard to its fatty acid make-up, but likewise with respect to its content of phospholipids. The fatty substances in the tumor contain, as calculated from their phosphorus content, 8.2 per cent of phospholipids, while the adhering fat contained only 0.45 per cent of such materials. The amount of phospholipids in normal human subcutaneous fat is almost a negligible quantity. The highest value obtained in an unpublished investigation by one of the authors was only 0.04 per cent, a value which compares favorably with that reported by others. The tumor fat, therefore, contains 200 times as much of phosphorized fat as does the subcutaneous fat. It is interesting to note that the adhering fat differs from the normal subcutaneous fat of man in that it contains almost 12 times as much of phospholipids. Whether this is a true difference, or merely the result of infiltration from tumor tissue, cannot be stated with certainty. Every possible precaution was

employed during the separation of the two tissues. Macroscopically, at least, the writers feel assured of a clear cut isolation, but since no histological examinations were made the possibility of microscopical contamination cannot be ruled out. It might seem, therefore, that the tissue surrounding the tumor contains lipids having more of the lecithins than does normal human fat. Similarly it apparently contains more cholesterol that does normal subcutaneous fat. An analysis of the fatty substances in the adhering material showed the presence of 0.97 per cent of the sterol, in other words practically 4 times as much as in the more normal fat. The tumor fat on the other hand contains approximately 200 times as much as does the normal fat. More than half (51 per cent) of the lipids in the pathological tissue is in the form of non-saponifiable matter. By mere fractionation of this matter it was possible to obtain an amount of pure cholesterol corresponding to 33 per cent of the total lipids. An exact determination of the sterol content of the tumor, as made by the digitonin method, revealed the presence of 8.59 per cent of cholesterol in this tissue. Calculated on the basis of the total lipids there was 48.81 per cent of cholesterol in the fatty substances of the tumor. In our previous investigations (1, 2, 5) we found the average cholesterol content of the lipids in xanthomata to be only 14.4 per cent of the total lipids. While we are not in a position to explain the presence of the larger amounts of cholesterol in the tumor studies in this investigation, we do, however, feel that a purpose will be served by recording observations which indicate to us that there is an anatomical difference between the large tumor and those previously reported on by us. On examining the former we were at once struck by the fact that it differed from the others in that it contained little fibrous tissue. Furthermore it was found to be apparently devoid of blood vessels, was markedly pedunculated, and had been sequestered for a long time. On the other hand, those previously studied by us were all of more recent origin, were not pedunculated, and contained blood vessels. Whether the anatomical difference is the cause of the high cholesterol content of the tumor analyzed here cannot be stated. We merely report the difference with the hope that the information may be of value in the future.

Several analyses were made of the blood of the individual from

whom the tumor was removed. The average cholesterol content was found to be 245 mg. per cent. This amount is higher than what is considered to be the normal cholesterol content of the blood. It might appear therefore that there is some relationship between the blood lipids and those of the tumor. This is not borne out from our previous observations, inasmuch as the blood cholesterol values were found to vary greatly. Some were within the normal limits, others were markedly high, and the rest just a bit above what is considered to be the normal blood cholesterol content, *e.g.* 148, 816, 650, 110, 160, and 640 mg. per cent. In the case reported here the blood contained 1187 mg. per cent of total lipids and 380 mg. per cent of phospholipids. Normally one finds approximately 30 per cent of the blood lipids as cholesterol and more than 50 per cent as phospholipids. In this respect the percentages of the lipids mentioned above are smaller in the blood of our patient than in the blood of the normal individual. As has already been mentioned the lipids in the tumor contain 200 times as much cholesterol and phospholipids as does normal human subcutaneous fat. It is apparent therefore that there is no direct parallelism between the nature of the blood lipids and those of the tumor. This is in agreement with our previous findings (1, 2, 5).

While it has thus not been possible to demonstrate a parallelism between the blood lipids and those of the tumor there is perhaps some relationship between the physiological activity of the three kinds of tissues (normal subcutaneous fatty, tumor, and adhering tissue) and the nature of the lipids in those tissues. During recent years considerable evidence has accumulated which indicates that the nature of the lipids, particularly the phospholipids, in a tissue varies with the activity of that tissue. No purpose would be served by a review of the literature on this subject since this has so recently been given by Bloor and his coworkers (6). Of the tissues studied in our investigation the tumorous tissue is probably the most active. Hence one should expect to find a greater content of the phospholipids in the fatty substances of the abnormal tissue. That this is the case is quite evident from our results. While it might appear, from a casual glance at the results in Table I, that the lipids in the tumor may be characterized by their high content of cholesterol, it is important to call attention again to the fact that the ratio of the cholesterol content in the lipids of normal

subcutaneous human fat to that present in the lipids of the tumor is practically the same as the ratio between the phospholipid content of the same two types of lipids. Whether this ratio will be maintained in all cases cannot be stated at this time, inasmuch as this is the first occasion we have had to determine the phospholipid content of the fatty substance in xanthomatous tumors. In this connection it is interesting to note that Bloor (7) has recently reported that the content of cholesterol and phospholipids of tumors varies with their physiological activity. Thus malignant tumors contain about 3 times the content of phospholipids and twice the amount of cholesterol as do benign tumors. Unfortunately Bloor did not report on the actual content of cholesterol and phospholipids in these tumors and for that reason it is, of course, impossible to make a comparison.

From a consideration of the facts stated above we suggest, therefore, that it is the nature of the tissues involved more than the nature of the blood lipids which determines the lipid make-up of xanthomata.

SUMMARY.

1. The nature of the lipids in a xanthomatous tumor has been determined and compared with that of the lipids in the tissue adhering to the tumor and with those in normal subcutaneous fat.

2. The fatty acid make-up of the lipids in the tumor differs markedly from that of these in normal subcutaneous fat and the lipids in the tissues adhering to the tumor. On the other hand, the nature of the fatty acids in the adhering fat is like that of normal subcutaneous fat.

3. The percentage of cholesterol and phospholipids in the total lipids of the tumor was found to be 48.81 and 8.1 per cent respectively. This is 200 times the amounts previously found for normal human subcutaneous fat.

4. The percentage of cholesterol in this tumor is considerably higher than the amount previously reported by us for other xanthomata. In accordance with our previous findings, however, no parallelism between the nature of the blood lipids and those of the tumor could be demonstrated.

5. It is suggested that the nature of the lipids in xanthoma is more dependent upon the activity of the tissue than on the nature of the blood lipids.

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THE INFLUENCE OF pH AND SOLUTION CONCENTRATION ON THE SURFACE TENSION OF GELATIN SOLUTIONS DETERMINED BY THE SESSILE BUBBLE METHOD.

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The present paper presents data, obtained by the sessile bubble method of measuring surface tensions, which define more clearly than heretofore the relationship between the surface tension of gelatin solutions at equilibrium and their concentration and pH, and show the existence of a condition of equilibrium between the gelatin adsorbed at the interface and the main bulk of the solution similar to that existing in the case of solutions of surface-active substances of low molecular weight. These data further emphasize the difficulty of obtaining and interpreting surface tension measurements of protein solutions in general.

There are two types of phenomena to be distinguished as a consequence of the surface concentration of proteins. It is well known that when such proteins as egg albumin are adsorbed at a solution-gas interface the protein becomes denatured and forms a solid surface film. Thus with the formation of a new phase, such as is represented by a solid surface film, it is difficult to consider the existence of a condition of equilibrium between the surface excess and the main bulk of the solution. Under such conditions it would seem irrational to attempt to measure the surface tension of a solution under conditions of surface equilibrium. It would appear equally difficult to give a proper interpretation to any values obtained intermediate to the time of forming an interface and that at which surface concentration had proceeded to the point where no further measurable change takes place. The formation of solid surface films formed by surface adsorption must

not be confused with such solid films as are sometimes formed by surface evaporation.

The other case to be considered is that in which the protein adsorbed at the interface is apparently as much in equilibrium with the bulk of the solution as is the surface excess in the case of substances of low molecular weight. In such cases it is desirable to attain a condition where a state of equilibrium is at least approached if it cannot be entirely reached. The rate of surface concentration of proteins is so slow and variable that it is not possible to compare the surface tension of one solution with that of another at an intermediate stage.

Gelatin solutions at 37° do not form solid surface films. The rate of surface adsorption of gelatin, even from 1 per cent solution, is so slow that it may require more than 24 hours to reach a state where no further change in surface tension takes place. Since the rate of surface concentration varies under different conditions, results obtained previous to those obtained when surface equilibrium has been established present an entirely different picture from results obtained at apparent equilibrium. The change of surface tension with time will sometimes give rise to a succession of maxima and minima in the curves which represent this change. This was found to occur when measuring the surface tension of solutions of gelatin (1) and sodium oleate (2) by the capillary rise method. Harkins and Harkins (3) have noted such a minimum 7 minutes after the formation of an interface in the case of blood serum and have chosen this minimum value as the true surface tension value for serum and attribute a further change in surface tension value to the formation of a solid film. It was shown previously (4) that the change of surface tension with time in the case of serum proceeds over a much longer period of time than 7 minutes. Recent experiments in this laboratory with the sessile bubble method show that there is a continuous decrease over a period of about 24 hours without the appearance of a solid surface film. It would, therefore, seem that the practice of choosing a value so far short of an equilibrium value would not be justified. If there were a possibility of the formation of a solid phase by surface adsorption at any period during the process of surface concentration as they suggest, it would not seem proper to use any value obtained under such conditions as a representative

surface tension value, for it is not likely that such a first minimum would always appear at the same place nor is there any reason to assume that it has the significance which Harkins and Harkins claim for it.

Not many of the large number of surface tension methods are adaptable to the measurement of surface tension under conditions

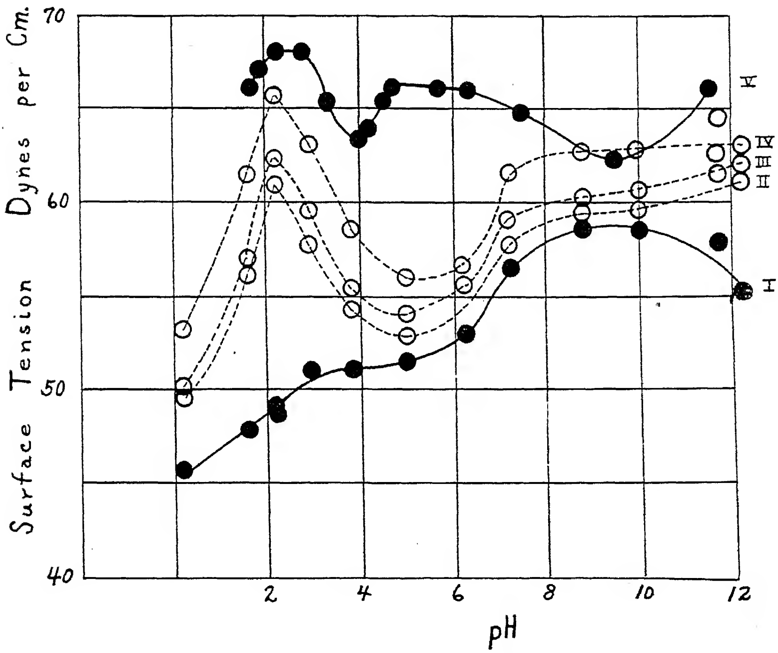

FIG. 1. The relationship of surface tension to pH in the case of 1 per cent gelatin solutions, at apparent equilibrium at points 1 (Curve IV), 2 (Curve III), 3 (Curve II), and 24 (Curve I) hours after the formation of an interface, as found by the sessile bubble method. The data of Curve V were obtained by Sugden's method.

where a state of equilibrium is arrived at so slowly. The reasons for choosing the sessile bubble method for measuring the surface tension of colloidal solutions are given in a previous paper (2).

The surface tension of gelatin solutions has been determined by a number of methods. The stalagmometer is still extensively used for measuring the surface tension of this type of colloidal solution.

That this method barely allows surface concentration to begin is illustrated by the data of Lasnitzki and Loeb (5) who found the surface tension of gelatin solutions varying in concentration from 0.3 to 1.0 per cent to be little different from that of water.

Sugden's method, as used by St. Johnston and Peard (6) for measuring the surface tension of gelatin solutions under varied

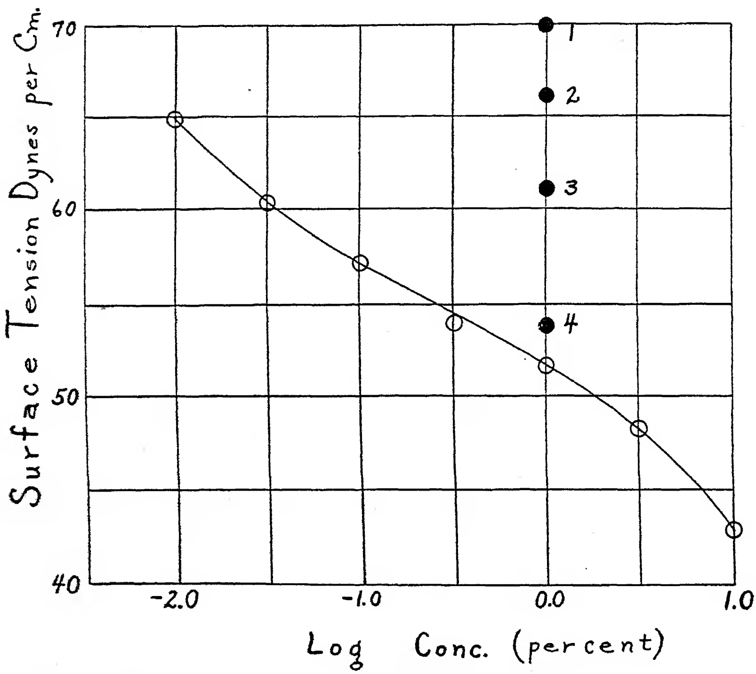

FIG. 2. The open circles represent data obtained by the sessile bubble method and show a relationship between surface tension and solution concentration similar to that shown in the case of substances of low molecular weight. The solid circles represent surface tension data obtained with 1 per cent solutions of gelatin by other methods: (1) by means of a stalagmometer, (2) by Sugden's method, (3) by the capillary rise method after 1 hour, and (4) by the drop weight method after half an hour.

conditions of concentration and pH, also gave results which are far above those obtained when a longer time is allowed for surface concentration to take place. The different effect upon surface tension to be ascribed to a change in pH when using this method

from that to be observed when using a static method is to be noted by comparing Curve V of Fig. 1 with Curves I to IV which represent data obtained by the sessile bubble method.

Data which were obtained an hour after the formation of an interface with 1 per cent gelatin solutions of varied pH, by the capillary rise method (1), are more nearly like those represented by Curve IV of Fig. 1, which also represents the change of surface tension with a change in pH, but they are somewhat higher. Data which were obtained $\frac{1}{2}$ hour after the formation of an interface with gelatin solutions of varied concentration by the drop weight method (1), approach more nearly the equilibrium values represented by the curve of Fig. 2. Values obtained 10 minutes after the formation of an interface, however, were found to fall far short of equilibrium values.

The data of the sessile bubble method when calculated as in the present experiments do not give absolute values but values which are relatively too high. (The surface tension of water expressed in absolute values when thus determined by the sessile bubble method is well known to be about 6 dynes too high.) It is believed, however, that they are *relatively* more accurate and represent more nearly a true picture of the effect of a varied solution concentration and pH than do readings obtained by other methods.

It is usually thought that the surface tension of colloidal solutions of surface-active substances is independent of solution concentration. That there is, however, a definite relationship between the surface tension of colloidal solutions and their concentrations was shown in the case of soap solutions (7) over a wide range of concentrations. That there is a definite relationship between the surface tension and solution concentration of gelatin solutions comparable to that of solutions of surface-active substances of low molecular weight is shown by the data represented in Fig. 2.

The manner in which the surface tension of 1 per cent gelatin solutions varies at equilibrium when the pH is varied over a wide range is shown by Curve I of Fig. 1. Curves IV, III, and II represent data obtained 1, 2, and 3 hours after the formation of an interface. The equilibrium values of Curve I were obtained 18 to 24 hours after the formation of an interface. In the case of solutions which contained hydrochloric acid, a longer time was required

for surface equilibrium to be established than in those cases where sodium hydroxide had been added. That an entirely different variation is represented when surface equilibrium has been reached from that to be observed at some period shortly after the formation of an interface is illustrated by a comparison of Curve I with the other curves of Fig. 1. To what extent possible hydrolysis prolongs the time required for surface equilibrium to be reached cannot be stated. Even when no acid or base was added and the possibility of hydrolysis was excluded the time required for surface equilibrium was far in excess of that represented by Curve II of Fig. 1, which represents data obtained 4 hours after the formation of an interface.

The gelatin used in these experiments was rendered ash-free and the pH determination[1] was made as described previously (8). 1 per cent solutions had a pH of 4.95. The apparatus used was the same as that used in measuring the surface tension of sodium oleate solutions by the sessile bubble method (7). In the present experiments the size of the sessile bubble was more carefully controlled so that its diameter varied from 45.5 to 47 mm. when surface equilibrium had been reached; *i.e.*, when no further change could be observed over a period of several hours. The temperature at which measurements were made was 37° ± 0.03°.

SUMMARY.

The sessile bubble method has been used to determine the surface tension of gelatin solutions under varying conditions of solution concentration and pH. The manner in which the surface tension changes with solution concentration indicates that proteins which have been adsorbed at a liquid-gas interface are probably in equilibrium with the bulk of the solution much as surface-active substances of low molecular weight are. It has been shown that a much longer time is required for apparent surface equilibrium than is usually allowed and that a period in excess of 24 hours is sometimes required for such a condition of equilibrium to be reached, emphasizing the need of a truly static method for measuring the surface tension of protein solutions.

[1] The writer gratefully acknowledges his indebtedness to his colleague, Dr. I. P. Earle, for carrying out the pH determinations.

It has been found that the picture represented by a comparison of surface tension data obtained at various pH values is on the whole quite different when readings are taken after a condition of apparent equilibrium has been reached from that of a comparison of data obtained within 3 or 4 hours after the formation of an interface. Between a range of pH 5 and 9, however, the relative change of surface tension with a change in pH is nearly the same at all stages.

It has been found that in general the addition of a base increases the surface tension of 1 per cent gelatin solutions and that the addition of an acid decreases it. A maximum value was found at a pH of 9. The usual minimum previously observed near the isoelectric point and the maximum occurring on the acid side of this point are lacking in the curve representing equilibrium values, although these are found in the curves representing data obtained 1, 2, and 3 hours after the formation of an interface. It is not known to what extent acid hydrolysis is responsible for this deviation.

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COMPOUND AMINO ACIDS.*

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INTRODUCTION.

A well known property possessed by amino acids is their ability to combine with acids and with bases. The change in acidity which takes place when such a reaction occurs is usually expressed graphically as a titration curve. From such curves the acidic and basic dissociation constants of amino acids can be calculated. On examination of the constants of the naturally occurring amino acids it is seen that they fall into three groups: (*a*) those having predominantly acidic properties, (*b*) those having predominantly basic properties, (*c*) those whose acidic and basic properties are about equal.

It follows that when an amino acid with predominantly acidic properties is added to an amino acid with predominantly basic properties a change in the acidity of the solution should take place to a measurable degree and this change should proceed in accordance with the law of mass action. The result of this reaction should be the formation of a salt which contains the two amino acids in the molecule ($RCOONH_3R'$). The chemical stability of such a salt appears to be a function of the dissociation constants of the two groups involved in the linkage. However, this type of compound differs from the usual linkage (—COHN—) when two amino acids unite to form a peptide. The stability of the peptide linkage as pointed out by Levene and Simms (1) is also a function of the dissociation constants of the two groups involved in the linkage.

With the exception of the recent publication of Han (2), who

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pointed out that histidine glutamate is probably formed in the isolation of glutamic acid, no work dealing with compound amino acids has apparently been carried out.

In the present investigation we have studied the formation of compound amino acids by following the changes in acidity which take place when an acidic amino acid such as glutamic acid is added to a basic amino acid such as arginine.

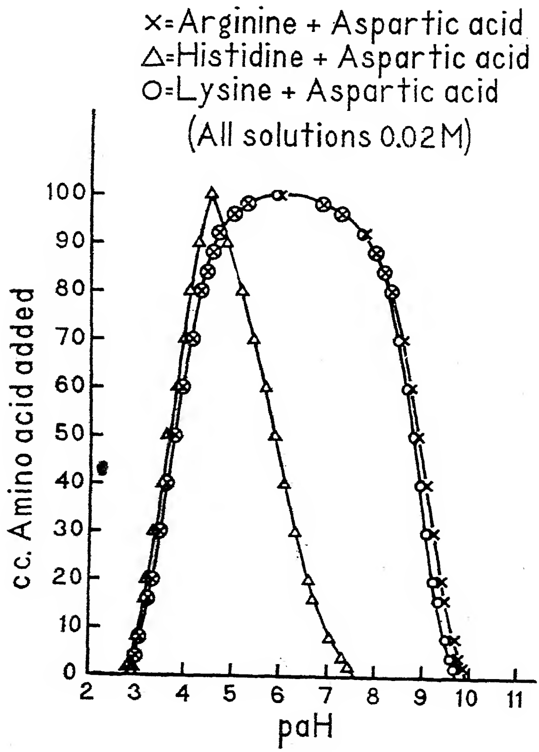

FIG. 1. Titration curves of aspartic acid.

EXPERIMENTAL.

The technique employed in obtaining the titration curves was similar to that described by Kirk and Schmidt (3). The amino acids employed were either prepared in this laboratory or were

commercial products. They were repeatedly recrystallized to insure a high degree of purity. Histidine and lysine were obtained as the dichlorides, arginine as the monochloride, and ornithine as the mixed chlorides. Glutamic and aspartic acids were the free acids. The hydrochlorides of the basic amino acids were neutralized by the addition of an equivalent amount of 0.1 N NaOH. To

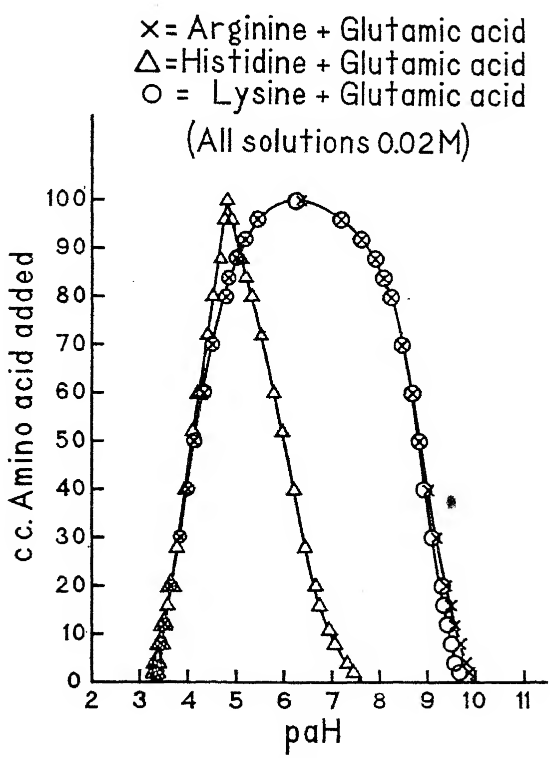

FIG. 2. Titration curves of glutamic acid.

5 cc. of the solution of basic amino acid in a 10 cc. flask varying amounts of glutamic or aspartic acid were added, and, *vice versa*, varying amounts of basic amino acid were added to a constant amount of acidic amino acid and the volume brought to 10 cc. by addition of carbon dioxide-free water. The solutions were 0.02 M with respect to each of the amino acids. The hydrogen ion activity of each solution was determined electrometrically.

The titration curves are represented graphically in Figs. 1 to 3. We have included a titration curve of ammonia with acetic acid as an illustration of the titration curve of a weak acid with a weak base and a curve of lysine with acetic acid. The titration curves of arginine and lysine with glutamic acid are essentially the same,

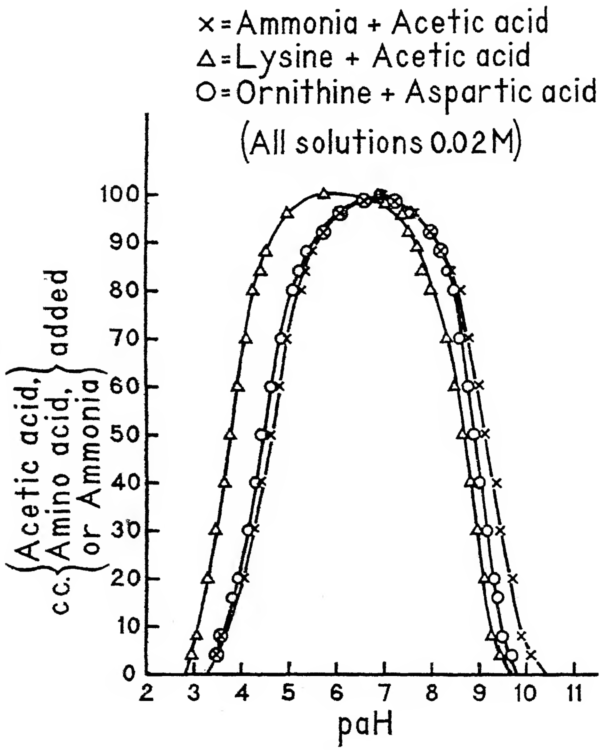

FIG. 3. Titration curves of acetic and aspartic acids.

while a similar relation holds for arginine and lysine with aspartic acid. The curves which histidine forms with aspartic and glutamic acids respectively are similar but differ from the curves first mentioned. The titration curve of ornithine with aspartic acid is essentially the same as the titration curve of acetic acid and ammonia.

DISCUSSION.

In Figs. 1 to 3 the point of equivalence indicating complete salt formation is shown by the abrupt change in the acidity of the solution on addition of a small amount of either amino acid in excess of an equivalent. The histidine curves do not show the distinct break in the curves. This is due to the fact that histidine is a relatively weaker base than lysine or arginine.

Since the second dissociation constants of both the basic and the acidic amino acids are very much smaller than the primary constants, the assumption can be made that for the purpose of forming compound amino acids the dicarboxylic amino acid acts as R—COOH, and the diamino acid acts as $R—NH_2$.

The hydrogen ion concentration in the salt solution can be calculated with the aid of an equation which is derived as follows: It is assumed that both the acid and the base which form the compound amino acid are weak electrolytes and obey the law of mass action. The hydrolysis reaction can be written

$$\overset{-}{A}\overset{+}{B} + H_2O \rightleftarrows HA + BOH$$

If 1 mol of salt is contained in V liters and X = the fraction hydrolyzed, then at equilibrium $\frac{1-X}{V}$ mols of salt will be practically completely dissociated; *e.g.*, $\frac{1-X}{V}$ = the concentration of the ions $\overset{-}{A}$ and $\overset{+}{B}$. $\frac{X}{V}$ denotes the concentration of acid which is assumed to be very slightly dissociated but relatively more so than the base. Now

$$(\overset{+}{H})\ (O\overset{-}{H}) = K_w$$

$$\frac{(\overset{+}{H})\ (\overset{-}{A})}{(HA)} = K_a$$

$$\frac{(\overset{+}{B})\ (O\overset{-}{H})}{(BOH)} = K_b$$

These when combined give

$$\frac{(\mathrm{HA})\ (\mathrm{BOH})}{(\overset{+}{\mathrm{B}})\ (\overset{-}{\mathrm{A}})} = \frac{K_w}{K_b\,K_a} = K_{\mathrm{H}}$$

Now

$$(\mathrm{HA}) = \frac{X}{V} \text{ and } (\mathrm{BOH}) = \frac{X}{V}$$

Hence

$$K_a = \frac{(\overset{+}{\mathrm{H}})\ (1 - X)}{(X)},\ K_b = \frac{(\overset{-}{\mathrm{OH}})\ (1 - X)}{X},\ \text{and } K_{\mathrm{H}} = \frac{X^2}{(1 - X)^2}.$$

Therefore

$$K_a = (\overset{+}{\mathrm{H}})\ \frac{1}{(K_{\mathrm{H}})^{\frac{1}{2}}},\ \text{or } \overset{+}{\mathrm{H}} = K_a\ (K_{\mathrm{H}})^{\frac{1}{2}},\ \text{and pH} = \tfrac{1}{2}\ \mathrm{pK}_a\ \mathrm{pK}_{\mathrm{H}}$$

K_a and K_b are the acidic and basic dissociation constants respectively of the acidic and basic amino acid, K_w is the dissociation constant of water, and K_{H} is the hydrolysis constant of the compound amino acid.

The ionization constant (K_s) of a compound amino acid can be calculated with the aid of the following equations:

$$\begin{array}{ccc}
\mathrm{RCOOH} & & \mathrm{R'NH_2} \\
(\mathrm{HA}) & & (\mathrm{BOH}) \\
\uparrow\downarrow & & \uparrow\downarrow \\
\mathrm{RC\overset{-}{O}O} + \overset{+}{\mathrm{H}} & & \mathrm{R'\overset{+}{N}H_3} + \overset{-}{\mathrm{OH}} \\
(\overset{-}{\mathrm{A}} + \overset{+}{\mathrm{H}}) & & (\overset{+}{\mathrm{B}} + \overset{-}{\mathrm{OH}}) \\
 & \uparrow\downarrow & \\
 & \mathrm{RCOO\ NH_3R'} & \\
 & (\mathrm{BA}) &
\end{array}$$

(1) $$(\overset{+}{\mathrm{H}})\ (\overset{-}{\mathrm{OH}}) = K_w$$

(2) $$\frac{(\overset{+}{\mathrm{B}})\ (\overset{-}{\mathrm{OH}})}{(\mathrm{BOH})} = K_b$$

(3) $$\frac{(\bar{A})\ (\overset{+}{H})}{(HA)} = K_a$$

(4) $$\frac{(\bar{A})\ (\overset{+}{B})}{AB} = K_s$$

(5) $$\overset{+}{B} + \overset{+}{H} = O\bar{H} + \bar{A}$$

(6) $$\Sigma \text{ acid} = HA + \bar{A} + BA$$

(7) $$\Sigma \text{ base} = BOH + \overset{+}{B} + BA$$

(6)-(7) $$\Sigma \text{ acid} - \Sigma \text{ base} = (HA) + (\bar{A}) - (BOH) - (\overset{+}{B}) \qquad \text{(a)}$$

(a)-(5) $$\Sigma \text{ acid} - \Sigma \text{ base} + (O\bar{H}) - (\overset{+}{H}) = (HA) - (BOH)$$

or

$$(BOH) = \Sigma \text{ base} - \Sigma \text{ acid} - (O\bar{H}) + (\overset{+}{H}) + HA \qquad \text{(b)}$$

from equation (5)

$$(\overset{+}{B}) = \bar{A} + O\bar{H} - \overset{+}{H} \qquad \text{(c)}$$

If equations (b) and (c) are substituted in equation (2)

$$K_b = \frac{(\bar{A} + O\bar{H} - \overset{+}{H})\ (O\bar{H})}{\Sigma \text{ base} - \Sigma \text{ acid} - (O\bar{H}) + (\overset{+}{H}) + (HA)} \qquad \text{(d)}$$

from equation (3)

$$(\bar{A}) = K_a \frac{HA}{\overset{+}{H}} \qquad \text{(e)}$$

If this is substituted in equation (d)

$$K_b = \frac{\left(K_a \dfrac{(HA)}{(\overset{+}{H})} + O\bar{H} - \overset{+}{H}\right)(O\bar{H})}{HA - \Sigma \text{ acid} + \Sigma \text{ base} + \overset{+}{H} - O\bar{H}} \qquad \text{(f)}$$

or

$$HA = \frac{(O\bar{H})^2 - K_w - K_b\,(\Sigma \text{ base} - \Sigma \text{ acid} + \overset{+}{H} - O\bar{H})}{K_b - K_a \dfrac{O\bar{H}}{\overset{+}{H}}} \qquad \text{(g)}$$

If this is substituted in equation (e)

$$(\overset{-}{\mathrm{A}}) = \frac{K_a}{\overset{+}{\mathrm{H}}}\left(\frac{(\overset{-}{\mathrm{OH}})^2 - K_w - K_b\,(\Sigma\text{ base} - \Sigma\text{ acid} + \overset{+}{\mathrm{H}} - \overset{-}{\mathrm{OH}})}{K_b - K_a\dfrac{\overset{-}{\mathrm{OH}}}{\overset{+}{\mathrm{H}}}}\right) \tag{h}$$

From equation (6)

$$\mathrm{BA} = \Sigma\text{ acid} - (\mathrm{HA} + \overset{-}{\mathrm{A}}) \tag{i}$$

Substituting equations (g) and (h) in equation (i), we get

$$\mathrm{A} = \Sigma\,\text{acid} - \left(\left(1 + \frac{K_a}{\overset{+}{\mathrm{H}}}\right)\frac{(\overset{-}{\mathrm{OH}})^2 - K_w - K_b\,(\Sigma\text{ base} - \Sigma\text{ acid} + \overset{+}{\mathrm{H}} - \overset{-}{\mathrm{OH}})}{K_b - K_a\dfrac{\overset{-}{\mathrm{OH}}}{\overset{+}{\mathrm{H}}}}\right) \tag{j}$$

If equation (h) is substituted in equation (c)

$$\overset{+}{\mathrm{B}} = \overset{-}{\mathrm{OH}} - \overset{+}{\mathrm{H}} + \frac{K_a}{\overset{+}{\mathrm{H}}}\left(\frac{(\overset{-}{\mathrm{OH}})^2 - K_w - K_b\,(\Sigma\text{ base} - \Sigma\text{ acid} + \overset{+}{\mathrm{H}} - \overset{-}{\mathrm{OH}})}{K_b - K_a\dfrac{\overset{-}{\mathrm{OH}}}{\overset{+}{\mathrm{H}}}}\right) \tag{k}$$

Substituting equations (h), (j), and (k) in equation (4) and setting

$$\frac{(\overset{-}{\mathrm{OH}})^2 - K_w - K_b\,(\Sigma\text{ base} - \Sigma\text{ acid} + \overset{+}{\mathrm{H}} - \overset{-}{\mathrm{OH}})}{K_b - K_a\dfrac{\overset{-}{\mathrm{OH}}}{\overset{+}{\mathrm{H}}}} = S \quad .$$

we get

$$K_s = \frac{\left(\overset{-}{\mathrm{OH}} - \overset{+}{\mathrm{H}} + \dfrac{K_a}{\overset{+}{\mathrm{H}}}S\right)\dfrac{K_a}{\overset{+}{\mathrm{H}}}S}{\Sigma\text{ acid} - \left[\left(1 + \dfrac{K_a}{\overset{+}{\mathrm{H}}}\right)S\right]}$$

In Table I numerical values for K_s, K_{H}, and pH of the compound amino acids studied are presented. K_a and K_b refer respectively

TABLE I.*

Constants Relating to Compound Amino Acids.

	K_a	K_b	K_H	pH		K_s
				Experimental.	Calculated.	
Arginine aspartate	2×10^{-4}	1.1×10^{-5}	4.55×10^{-6}	6.00	6.37	3×10^{-2}
Histidine "	2×10^{-4}	1.01×10^{-8}	5.00×10^{-3}	4.50	4.85	1.8×10^{-2}
Lysine "	2×10^{-4}	0.99×10^{-5}	5.00×10^{-6}	5.88	6.34	4×10^{-2}
Ornithine "	2×10^{-4}	4.46×10^{-6}	1.12×10^{-5}	5.76	6.17	1.7×10^{-1}
Arginine glutamate	5.62×10^{-5}	1.10×10^{-5}	1.62×10^{-5}	6.45	6.65	3×10^{-2}
Histidine "	5.62×10^{-5}	1.01×10^{-8}	1.78×10^{-2}	4.81	5.13	1.5×10^{-2}
Lysine "	5.62×10^{-5}	0.99×10^{-5}	1.78×10^{-5}	6.32	6.63	4×10^{-2}
Lysine acetate	1.84×10^{-5}	0.99×10^{-5}	5.55×10^{-5}	6.98	6.87	2×10^{-2}
Ammonium acetate	1.84×10^{-5}	1.81×10^{-5}	3.00×10^{-5}	7.00	6.96	2.5×10^{-2}

* With the equation given by Washburn instead of ours the calculated pH values do not differ more than 0.05 from our calculated values. (Washburn, E. W., Introduction to the principles of physical chemistry, New York and London, 318 (1915).)

to the dissociation constant of the predominant group of each of the amino acids in the compound. With the exception of the basic dissociation constant of arginine which is an unpublished value, the data have been taken from the tables of Kirk and Schmidt (4). The data given under pH indicate a satisfactory agreement between the calculated and experimentally determined values of acidity in solutions of the compound amino acids. The calculated ionization constant (K_s) for ammonium acetate, with molalities instead of activity values, varies but little over the whole range of the curve and the average value checks with that obtained from conductivity measurements. For the amino acids data for the activity coefficients are not available. With molalities for the purpose of calculating ionization constants, considerable variation is shown over the range of the titration curve. This seemingly indicates that the activities of the amino acids may vary considerably with varying concentration. The ionization constants given in Table I are the averages of the values which appeared to us as most probable.

In the present investigation only amino acids having pronounced acid and basic dissociation constants have been used for the study of compound amino acids since changes in the acidity of the solution can be most readily followed. The salt formation can, however, be extended to all amino acids having differences in dissociation constants. The stability of salts formed from amino acids having dissociation constants which are small is necessarily not great.

Hardy's (5) observation that many of the protein constituents of tissues or tissue fluids do not exist as such but are bound up in complexes is of importance in connection with this study. Compound proteins which are formed by the interreaction of acidic and basic proteins have been studied by a number of investigators. Hunter (6) and Gay and Robertson (7) prepared a number of compound proteins. Schmidt (8) demonstrated the formation of compound proteins by following the changes in acidity which take place when a predominantly acidic protein is added to a predominantly basic protein.

The formation of compound amino acids and compound proteins is probably not unlike the union of toxin and antitoxin and other immune bodies. Thus Arrhenius and Madsen (9) have shown that

the law of mass action can be applied to the union of tetanus lysin and antilysin and this reaction is similar to that which takes place between ammonia and boric acid. Gahl (10) has recently shown in this laboratory that the union of complement and amboceptor likewise follows the law of mass action.

SUMMARY.

1. By means of titration curves the changes of acidity on addition of a predominantly acidic amino acid to a predominantly basic amino acid have been followed.

2. It is shown that such amino acids can react to form compound amino acids.

3. Methods for the calculation of and data for the hydrolysis constants and ionization constants of certain compound amino acids are presented.

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NITROGENOUS SUBSTANCES IN ZINC FILTRATES OF HUMAN BLOOD.

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It was reported in a recent paper by the writer that protein-free blood filtrates prepared by the Hagedorn-Jensen technique contain considerably smaller quantities of reducing non-sugars than tungstate filtrates, and that a new method of deproteinization by zinc yields filtrates practically free of such substances (1). In 1927 we suggested that " . . . . glutathione is responsible for a substantial fraction of the residual reduction in tungstic acid filtrates . . . . ," and at the same time demonstrated that thioneine (ergothioneine), another constituent of tungstate filtrates, also reduces the Shaffer-Hartmann reagent (2). One may infer from the correlation of the two observations that lower quantities of reducing non-sugars would go hand in hand with lower nitrogen contents in the several sorts of blood filtrates. Experimental evidence shows that such a parallelism actually exists; namely, zinc filtrates (free of reducing non-sugars) contain upward of 10 mg. per cent less nitrogen than tungstate filtrates, while the zinc hydroxide filtrates of Hagedorn and Jensen, which are midway between the two in regard to reducing non-sugars, occupy the same position in regard to their nitrogen content.

As reported in a preliminary note (3), our zinc filtrates do not consume iodine at acid reaction and give no color reaction with Benedict's uric acid reagent, facts indicating the absence of glutathione, thioneine, and uric acid. (Herbert and Groen recently demonstrated that filtrates prepared by the Hagedorn-Jensen method with zinc hydroxide and by the colloidal iron method of Michaelis and Rona, are also free of glutathione (4).) However, the normal quantities of glutathione, thioneine, and uric acid do not account for the entire difference between the

nitrogen contents of tungstate and zinc filtrates. For human blood 100 mg. of glutathione per 100 cc. of corpuscles (Hunter and Eagles) would represent about 6 mg. of non-protein nitrogen per 100 cc. of whole blood, on the basis of 13.68 per cent of nitrogen in glutathione (Hopkins, 1929). Thioneine, with a nitrogen content of 18.33 per cent, would account for about 1.5 mg. per cent of non-protein nitrogen on the basis of 7.5 mg. per 100 cc. as the thioneine content of whole blood (Benedict, 1929) or for nearly 3 mg. per cent according to the older figures of Benedict (1926). Adding 1 mg. per cent of uric acid nitrogen, we have a sum total of 8.5 to 10 mg. per cent of non-protein nitrogen, accounting for about two-thirds to three-fourths of the average difference between the nitrogen content of tungstate and zinc filtrates.

This difference prompted a more detailed study of the nitrogen content of zinc filtrates with the purpose of obtaining information as to their adequacy for blood analysis in the clinical laboratory, frequently involving the determination of non-protein nitrogen and individual nitrogenous substances, such as urea, uric acid, and creatinine. We feel that, since we are able to determine true blood sugar in zinc filtrates (1), it is difficult to justify the acceptance of total reduction (apparent sugar) values, as determined by older methods, for true sugar. But then arises the question as to whether it is necessary to prepare two separate filtrates: a zinc filtrate for the determination of sugar and a tungstate filtrate for the rest of the analysis, or whether it is possible to carry out adequately the determinations enumerated in a single zinc filtrate.

The first question to decide was: Do the non-protein nitrogen values in zinc filtrates permit the drawing of a border line between normal and pathologic conditions as clear cut as in tungstate filtrates? In order to obtain conclusive information, non-protein nitrogen determinations were carried out in both the tungstate and the zinc filtrates of a large number of blood samples, normal and pathologic. The twenty analyses recorded in Table I are illustrative of the results obtained in a series of close to 100 normal samples, nearly half of them mixtures of two or more individual blood specimens. The average values given at the end of Table I, therefore, actually represent averages of well over 100 normal individual cases.

In the tungstate filtrates we find the well known range of 23 to

35 mg. of nitrogen per 100 cc. of blood, with an average of 30 mg. per cent. The average in the zinc filtrates is 17 mg. with variations between 11 and 22 mg. per cent; it may be noted that only in three out of 50 cases did the figures run above 20 mg. per cent, and that the maximum of 22 mg. per cent occurred in but a single case.

The difference between the nitrogen content of tungstate and zinc filtrates, 11 to 16 mg. per cent, is noteworthy for the narrowness in its range of variation. This indicates that the amount of the nitrogenous substances which are precipitated by zinc but pass into the filtrates with tungstate precipitation, is rather uniform in normal human blood, so uniform in fact that it is possible to compute the non-protein nitrogen values of zinc filtrates from those of tungstate filtrates and *vice versa*, by the subtraction or addition, respectively, of the average difference of 13 mg. per cent.

TABLE I.

Non-Protein Nitrogen in Tungstate and in Zinc Filtrates of Normal Human Blood.

Experiment No.	Tungstate filtrate.	Zinc filtrate.	Difference.	Experiment No.	Tungstate filtrate.	Zinc filtrate.	Difference.
	mg. per cent	*mg. per cent*	*mg. per cent*		*mg. per cent*	*mg. per cent*	*mg. per cent*
1	30	14	16	41	35	20	15
2	31	18	13	42	31	18	13
3	27	15	12	43	28	16	12
4	31	17	14	44	30	18	12
5	34	21	13	45	31	18	13
6	35	21	14	46	28	15	13
7	28	14	14	47	34	18	16
8	33	17	16	48	28	17	11
9	29	15	14	49	28	15	13
10	27	14	13	50	34	20	14

	Tungstate filtrates.	Zinc filtrates.	Difference.
Lowest	23	11	11
Highest	35	22	16
Average	30.3	17	13.3

The twenty pathologic blood samples, presented in Table II, are examples from a series of more than forty analyses, the average

and the extreme values given in Table II having reference to the entire series. The lowest nitrogen value in the group is 21 mg. per cent for zinc filtrates, corresponding to 36 to 38 mg. per cent in tungstate filtrates. The range of differences between the two filtrates is considerably wider than in the normal cases, due more or less to an increased uric acid content, and in some cases possibly to other nitrogenous substances.

TABLE II.

Non-Protein Nitrogen in Tungstate and in Zinc Filtrates of Pathologic Blood Samples.

Experiment No.	Tungstate filtrate.	Zinc filtrate.	Difference.	Experiment No.	Tungstate filtrate.	Zinc filtrate.	Difference.
	mg. per cent	*mg. per cent*	*mg. per cent*		*mg. per cent*	*mg. per cent*	*mg. per cent*
1	104	83	21	31	38	24	14
2	45	27	18	32	134	118	16
3	52	34	18	33	46	33	13
4	39	24	15	34	139	115	24
5	38	25	13	35	188	171	17
6	75	61	14	36	163	142	21
7	39	28	11	37	183	161	22
8	39	28	11	38	128	113	15
9	38	21	17	39	46	33	13
10	200	185	15	40	40	28	12

	Tungstate filtrates.	Zinc filtrates.	Difference.
Lowest	36	21	11
Highest	200	185	24
Average			15.5

The information of main interest to be derived from these figures concerns the relation between the upper limit of non-protein nitrogen in the normal group and the lower limit in the pathologic cases. As disclosed by Tables I and II, the two values meet at 21 mg. per cent, with an overlapping of only 1 mg. per cent, so that the line of division between normal and pathologic cases is rather clear cut.

Further inquiry was extended as to the adequacy of zinc filtrates for the determination of some individual nitrogenous substances frequently involved in clinical blood analysis. Uric acid, as

pointed out before, is absent from zinc filtrates, consequently its determination necessitates a separate tungstate precipitation. What is the situation in regard to urea and creatinine, the other two substances of frequent interest, from the viewpoint of diagnosis? As to urea, it can be determined in zinc filtrates with the same facility as in tungstate filtrates. Comparative determinations, according to the figures in Table III, yield substantially

TABLE III.

Partition of Non-Protein Nitrogen in Tungstate Filtrates and in Zinc Filtrates.

The results are expressed in mg. per 100 cc. of blood.

Case No.	Non-protein N.		Urea N.		Creatinine.		Total creatinine.	
	Tungstate filtrates.	Zinc filtrates.	Tungstate filtrates.	Zinc filtrates.	Tungstate filtrates.	Zinc filtrates.	Tungstate filtrates.	Zinc filtrates.
1	30	14	11	10			3.6	3.4
2	31	18	13	13			3.9	3.3
J.S.	37	20	14	14			3.7	3.2
J.W.	35	22	18	19				
15	34	20	13	14				
26	32	19	15	15				
22	27	15	10	9			4.0	3.5
23	28	15	9	9			4.1	3.7
56	77	58	50	50			5.6	7.1
57	43	29	23	23				
66	99	84	78	71			6.9	6.1
60	80	67	60	55	6.0	7.2		
79	128	113	102	103	6.3	4.8	13.3	12.0
1246	142	127	114	113	14.7	12.0	22.0	19.2
1280	183	161	150	140(?)	22.8	19.4	29.0	25.0
1307	177	154	143	143	23.1	20.0	27.7	25.0
1314	144	132	120	123	21.0	19.5	26.0	22.8
1328	105	95	80	80	22.0	19.1	27.7	23.3
M.C.	189	182	176	182	14.8	13.3	24.5	20.0

identical results for the two kinds of filtrates, at low as well as at high concentrations of urea.

As regards creatinine and "total creatinine" (the sum of creatinine plus creatine), zinc filtrates yield consistently somewhat lower results than tungstate filtrates. The discrepancies—roughly proportional to the total amount—are quite insignificant at low creatinine values, and at normal levels are practically

obliterated by experimental errors. As to whether this is an indication of a partial loss of creatinine due to precipitation with zinc, would be difficult to conclude from the experiments on hand. It is conceivable that some substance other than creatinine, giving a similar color reaction, is removed by zinc. But this is, we believe, outside of our present problem. What we are concerned with is to decide whether or not the creatinine values in zinc filtrates are apt to furnish the information desired by the clinician. Comparative determinations in tungstate and zinc filtrates, given in Table III, imply an answer in the affirmative; differences as between 6.0 and 7.2 or 23 and 20 mg. per cent, are certainly of no consequence from the viewpoint of diagnosis or prognosis.

SUMMARY.

Blood filtrates prepared by deproteinization with zinc contain substantially smaller quantities of nitrogen than tungstate filtrates.

The non-protein nitrogen in zinc filtrates normally varies between 11 and 21 mg. per 100 cc. of human blood, the average being 17 mg. per cent: in cases of nitrogen retention the non-protein nitrogen is in excess of 21 mg. per cent.

Urea shows identical values in zinc and tungstate filtrates, while creatinine is slightly lower and uric acid entirely absent in zinc filtrates.

Zinc filtrates, prepared for the determination of true blood sugar are also adequate for the determination of non-protein nitrogen, urea, and creatinine.

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ON THE COLORIMETRIC ESTIMATION OF GUANIDINE BASES IN BLOOD.*

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In 1924 Tiegs reported a new color reaction for the guanidine bases. On the basis of this reaction Marston (1924) elaborated a reagent for the colorimetric estimation of guanidine bases in pure solution. This reagent consisted of an alkaline peroxide-ferrocyanide-nitroprusside mixture which is by no means specific for the guanidine bases. Uric acid, ammonia, and most reducing substances inhibit the color reaction. Creatine and creatinine produce a color with the reagent. A short time later Marston (1925) pointed out that in estimating the guanidine bases in tissues ammonia may be removed by permutit and uric acid by precipitation with silver lactate but no details of the method were given. Whitehorn (1923) had already shown, however, that permutit removes guanidine from an aqueous solution of guanidine hydrochloride, and Pfiffner and Myers (1926) observed that permutit also removes methylguanidine and *as*-dimethylguanidine from aqueous solution, although the removal is not quantitative. A study of the reagent was undertaken in this laboratory with the object of applying it to the colorimetric estimation of guanidine bases in blood. In a preliminary communication Pfiffner and Myers (1926) described a modified peroxide-ferrocyanide reagent which kept well and gave within 5 minutes a full color development and did not fade or become turbid for more than an hour. Weber (1927) described a reagent for the colorimetric estimation of guanidine bases in which he substituted potassium ferricyanide for the potassium ferrocyanide and hydrogen peroxide of Marston's reagent. This substitution doubled the sensitivity of the reagent to guanidine bases. Major and Weber (1927) applied the reagent to the colorimetric estimation of guanidine bases in blood, and have made several reports of their various observations dealing particularly with hypertension.

In connection with observations on the retention of creatinine in nephri-

* The data here presented are taken from a dissertation submitted by Joseph J. Pfiffner to the Graduate College of the State University of Iowa, June, 1928, in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

tis (Myers, 1914; Myers and Fine, 1914), it was pointed out that it was quite possible that this might be followed by transformation to the toxic methylguanidine. The following year Foster (1915) announced the isolation of a toxic substance from the blood of uremic patients as a gold salt although he made no statement as to its nature. At about this time some work was done by one of us (M.) in an effort to isolate methylguanidine from the blood of nephritic patients, but owing to apparently justified criticisms of the method employed (Baumann and Ingvaldsen, 1918; Greenwald, 1919) the work was abandoned.

With the appearance of the color reaction of Marston (1925) the work was resumed. Our findings (Pfiffner and Myers, 1926) indicated that the methylguanidine content of normal blood did not exceed 0.2 mg. per 100 cc., but the figures obtained on some pathological bloods were comparatively high. Although we had considered the question of the interference produced by creatinine, creatine, and urea, determined their color value and the quantities present in the various specimens, we ultimately concluded that we had underestimated the importance of these contaminants in the color reaction and decided to discard our results. Shortly after the publication of our preliminary report, Weber (1927) reported a study of the Marston color reaction, which has been followed by several papers by Major and Weber (1927) and Major (1929), in which they have presented considerable data showing that in patients with nephritis, and with arterial hypertension without nitrogen retention, there is a definite increase in the color reaction ascribed to the guanidines, the highest figures being found in the nephritic group.

In a very interesting and suggestive paper, Minot and Cutler (1928) have recently called attention to the presence of guanidine in the blood of dogs suffering from carbon tetrachloride and chloroform poisoning and to the antagonistic and beneficial action of calcium in this condition and in guanidine poisoning. With the development of the toxemia, a fall in the blood sugar and a rise in the icteric index was observed. In a subsequent preliminary report, Minot and Cutler (1929) have noted somewhat similar findings in acute liver injury and eclampsia.

Without entering into a further discussion of the subject, suffice it to say that there are a number of observations, in which a low blood calcium, and in some instances a low blood sugar, has been found to be associated with the presence of "guanidine compounds" in the blood, indicating the importance of this determination.

The object of the present paper is to record certain observations relative to the colorimetric reaction and to describe a modification of Weber's method which makes the use of a correction factor for blood creatine unnecessary.

The peroxide-ferrocyanide and the alkaline ferricyanide reagents have certain advantages and disadvantages. Both reagents pro-

duce an orange-red color with guanidine base[1] and a red color with methylguanidine[1] and *as*-dimethylguanidine.[1] With the peroxide-ferrocyanide reagent *as*-dimethylguanidine produces about 85 per cent of the color produced by methylguanidine, whereas guanidine produces 66 per cent. With the alkaline ferricyanide reagent *as*-dimethylguanidine produces 92 per cent of the color produced by methylguanidine, whereas guanidine produces only 40 per cent. These figures do not quite agree with those of Weber (1927) who found that guanidine and methylguanidine gave the same color value but dimethylguanidine only two-thirds as much. It appears fortunate that methylguanidine and *as*-dimethylguanidine have practically the same color value since it is expected that these two derivatives would be found in biological material rather than guanidine itself.

The alkaline ferricyanide reagent has the disadvantage of being very sensitive to the influence of salts. 20 mg. of sodium chloride depress the color development of 0.2 mg. of methylguanidine 20 per cent, whereas this amount of sodium chloride has no effect when the color is developed with the peroxide-ferrocyanide reagent. Ammonia and methylamine depress color development with both reagents. Color depression with ammonia is about 4 or 5 times greater with the peroxide-ferrocyanide reagent than with the alkaline ferricyanide reagent. The presence of excess alkali inhibits color development with either reagent. With the peroxide-ferrocyanide reagent the methylguanidine equivalent of 1 mg. of urea is 0.011; 1 mg. of creatinine, 0.005; 1 mg. of creatine, 0.22. With the alkaline ferricyanide reagent the methylguanidine equivalent of 1 mg. of urea is 0.006; 1 mg. of creatinine, 0.013; 1 mg. of creatine, 0.22. Time was allowed for full color development. Creatine develops a full color in about 5 minutes whereas urea and creatinine require about 15 minutes. The same color equivalents are displayed in the presence of a guanidine base.

[1] Standard solutions of guanidine hydrochloride, methylguanidine sulfate, and *as*-dimethylguanidine sulfate containing 1 mg. of the respective base per cc. were prepared by dissolving 160.2 mg. of guanidine hydrochloride, 167.1 mg. of methylguanidine sulfate, or 156.3 mg.of *as*-dimethylguanidine sulfate in distilled water and diluting to 100 cc. Diluted standards of the above three compounds were made by dilution with distilled water so that 10 cc. of solution contained 1 mg. of the respective base. Fresh standards were made up each week.

Both reagents react with arginine, histidine, and cysteine. Cystine gives no reaction. Arginine gives a rose-red color while histidine and cysteine give a green color. Histamine gives a green to reddish brown color depending on the amount present. Adrenalin and some of the alkaloids such as physostigmine, pilocarpine, and pelletierine give a red color. Procaine gives a green color. On the other hand methyl urea, β-methyl hydantoin, β-methyl hydantoic acid, sodium glycocholate, and thioneine do not give a color reaction.

The alkaline oxidation product of sodium nitroprusside responsible for color development with guanidine bases in the presence of ferricyanide can be obtained in dry stable form by precipitation from the oxidation mixture with ethyl alcohol. The method of obtaining the substance is as follows: Dissolve 5 gm. of sodium nitroprusside in 100 cc. of distilled water. Add 25 cc. of 40 per cent sodium hydroxide and while cooling in an ice bath add 30 per cent H_2O_2 slowly with constant stirring until a maximum green color is attained. Then add slowly 10 cc. more of 30 per cent H_2O_2. Transfer to a separatory funnel and add with shaking 700 cc. of 95 per cent ethyl alcohol. A heavy brown oil separates to the bottom. Draw off the precipitate and after dissolving in about 100 cc. of distilled water reprecipitate with the addition of 7 volumes of 95 per cent ethyl alcohol. After separating, dry the precipitate *in vacuo* over phosphorus pentoxide. A yellow amorphous water-soluble powder is obtained. The yield is about 5 gm.

In several different lots the nitrogen content varied from 9.5 to 11.5 per cent. If a few drops of a 1 per cent solution are added to a solution of a guanidine base (guanidine, methylguanidine, or *as*-dimethylguanidine) containing 0.05 mg. per cc., followed by a drop of 10 per cent potassium ferricyanide solution a deep red color develops immediately. No color is developed if ferrocyanide is used instead of ferricyanide. If the ferricyanide is added to the solution of the guanidine base *before* the oxidized nitroprusside product the color develops very slowly and requires about 1 hour for full color development compared to about 2 minutes when the reagents are added in the reverse order. If the 10 per cent potassium ferricyanide solution and the 1 per cent solution of oxidized nitroprusside product are first mixed in equal parts and then

added to the solution of a guanidine base there is no color development for 30 minutes and then a faint red color begins to develop. Sodium cyanide discharges the color of the reagents as well as the color formed by the interaction of the reagents with guanidine bases.

It would appear that the color formation is due to a reaction between the guanidine base, an alkaline oxidation product of sodium nitroprusside, and potassium ferricyanide in the order named. In the peroxide-ferrocyanide reagent the ferricyanide radical is apparently furnished by the oxidation of ferrocyanide, the alkaline peroxide oxidizing the sodium nitroprusside. In the alkaline ferricyanide reagent the nitroprusside is oxidized with alkaline ferricyanide and the ferricyanide radical having been added in excess is present in sufficient concentration for the color reaction.

In confirmation of Weber (1927) it was found that norit adsorbs guanidine bases quantitatively from a slightly alkaline solution and liberates them almost completely when treated with acidified alcohol. In a series of recoveries of guanidine bases from pure aqueous solution by adsorption on blood charcoal (Merck's purified by acid) under the conditions described by him we were able to recover methylguanidine on an average to the extent of 88.7 per cent (extreme variations 79 to 95 per cent), guanidine, 77.5 per cent (extreme variations 75 to 80 per cent), and *as*-dimethylguanidine 82.5 per cent (extreme variations 75 to 90 per cent). The adsorption is complete, low recoveries being accounted for because of incomplete release by acid alcoholic treatment. Norit does not adsorb guanidine base from neutral aqueous or absolute alcoholic solution although it does adsorb variable amounts of methylguanidine and *as*-dimethylguanidine under those conditions. Charcoal has the great disadvantage of adsorbing and releasing creatine under the same conditions as guanidine bases. Kaolin does not adsorb creatine and from this standpoint would be the ideal adsorbent, but it fails in that it does not adsorb the guanidine bases quantitatively. Adsorption of methylguanidine with Lloyd's reagent was only 75 per cent complete.

Since creatine is adsorbed by blood charcoal and released with acid alcoholic treatment it would interfere in the determination of guanidine bases in the blood and consequently Weber (1927)

introduced a correction factor for the amount of creatine present. This necessitated a determination of creatine and creatinine in the blood extract just prior to developing the color for the blood guanidine determination. The correction could not be based on the creatine content of the blood because, during the course of the determination, an acid solution of the blood extractives is concentrated on the water bath, converting some of the creatine to creatinine.

Since the obvious use of the method under discussion is to demonstrate the presence or absence of guanidine-like substances in the blood in certain pathological conditions it seems desirable to estimate the guanidine bases directly rather than by difference through the use of a correction factor for creatine. The use of a correction factor appears questionable due to the variation in percentage recovery of guanidine bases both from pure solution and from blood.

The logical solution is the conversion of creatine to creatinine by acid hydrolysis. It would be more convenient to autoclave the Folin-Wu filtrate before treatment with charcoal but the urea present would interfere. Urea is not adsorbed by charcoal in alkaline solution but autoclaving in acid solution would hydrolyze the urea and the ammonia formed would be adsorbed and subsequently released. Small amounts of ammonia interfere with color development. Hence in the described modification of Weber's method creatine is converted to creatinine after it is released from the charcoal. The percentage recovery with the modified direct technique is about the same (average 75.5 per cent) as the percentage recovery (average 76.2 per cent) obtained in a series of recoveries with Weber's (1927) method corrected for creatine. In our hands the variation in percentage recovery was the same in either case. It appears that some substance or substances are present in the final residue which tend to inhibit color development since guanidine bases added to the blood residue immediately before color development do not give their full color reaction. The chloride content of the residue is doubtless one factor since the average blood residue contains about 15 mg. of chloride calculated as sodium chloride. With the alkaline ferricyanide reagent 20 mg. of sodium chloride depress the color development of 0.2 mg. of methylguanidine as much as 20 per cent.

Method.

The modified method is as follows: 50 cc. of Folin-Wu blood filtrate are pipetted into a 150 cc. Erlenmeyer flask. 3 to 4 drops of 10 per cent NaOH are added followed by 0.5 gm. of blood charcoal (Merck's purified by acid). The contents of the flasks are mixed and filtered through a moist filter (Whatman No. 40). The flask is thoroughly drained. The filter is allowed to drain for

TABLE I.

Recovery of Methylguanidine from Blood.

Blood sample No. (1)	Methylguanidine added to 10 cc. of blood.* (2)	Methylguanidine as determined. (3)	Percentage recovery, uncorrected. (4)	Methylguanidine recovered, corrected.† (5)	Percentage recovery, corrected. (6)
	mg.	*mg.*		*mg.*	
1	0.40	0.34	85	0.32	80
	0.40	0.32	80	0.30	75
	0.20	0.16	80	0.14	70
2	0.10	0.10	100	0.08	80
	0.05	0.06	120	0.04	80
3	0.10	0.09	90	0.07	70
	0.08	0.075	94	0.055	69
	0.05	0.06	120	0.04	80
4	0.04	0.05	125	0.03	75
	0.06	0.06	100	0.04	67
	0.08	0.085	106	0.065	81
	0.10	0.11	110	0.09	90
Average			100.8		75.5

* Mixed human non-hypertensive pathological blood. Normal blood chemistry.

† Correction of 0.2 mg. of methylguanidine per 100 cc. of blood.

5 minutes and then returned to the original flask. 25 cc. of 95 per cent ethyl alcohol containing 0.5 cc. of N HCl are added to the contents of the flask. The flask is tightly stoppered, shaken, and allowed to stand overnight. The alcoholic solution is then filtered (Whatman No. 40). 20 cc. of the filtrate are transferred to an evaporating dish and brought to dryness on a water bath between

80–90°. The residue is taken up in 10 cc. of 0.2 N HCl, the dish covered with tin-foil (deep Pyrex dishes were used), and autoclaved for 30 minutes at 120°. The contents of the dish are again brought to dryness on a water bath between 80–90°. The last traces of HCl are dissipated by the addition of 1 or 2 cc. of absolute alcohol and subsequent evaporation. The residue is taken up in 4 cc. of distilled water and 1 cc. of alkaline ferricyanide reagent is added. Methylguanidine standards are made up simultaneously.

TABLE II.

"Guanidine" in Non-Hypertensive Blood Samples.

Subject.	Age.	Sex.	Blood chemistry. Uric acid.	Blood chemistry. Urea N.	Blood chemistry. Creatinine.	Methylguanidine.	Diagnosis.
	yrs.		*mg. per 100 cc.*	*mg. per 100 cc.*	*mg per 100 cc.*	*mg. per 100 cc.*	
E. W. M.	24	M.				0.2	Normal.
J. J. P.	24	"				0.3	"
P. W. S.	42	"				0.2	"
D. B. M.	28	"				0.2	"
C. L.	68	"	6.9	14.2	1.1	0.2	Perforating ulcer of duodenum.
E. T.	31	"	3.5	16.8	1.1	0.3	Psychoneurosis, neurosthenia.
S. B.	56	"	3.9	11.8		0.3	Psychoneurosis.
L. G.	40	F.	2.4	10.6	2.5	0.2	Neurosis, menopause.
C. R.	14	M.	3.4	13.0	2.0	0.2	Hematuria.
M. R.	44	F.	3.8	10.4	1.7	0.2	Chronic cystitis.
Average						0.23	

The solutions are centrifuged and color comparison made within the second 5 minutes after addition of the reagent.

A series of methylguanidine standards containing 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, and 0.2 mg. of methylguanidine base is made up in matched test-tubes (12 × 100 mm., and marked at the 4 cc. level) from a solution containing 1 mg. of methyl guanidine base per 10 cc. and diluted with distilled water to the 4 cc. mark. To each is added 1 cc. of the alkaline ferricyanide reagent. If by inspection the unknown indicates a concentration of methylguanidine greater than 0.07 mg., it is matched against a

0.2 mg. standard in a Bausch and Lomb micro colorimeter, and the amount of methylguanidine present determined by reference to a proportionality curve. If the unknown indicates a concentration of less than 0.07 mg. the amount present is estimated by inspection. The amount found represents the amount in 4 cc. of

TABLE III.

"Guanidine" in Hypertensive Blood Samples.

Subject.	Age.	Sex.	Blood chemistry.			Blood pressure.	Methylguanidine.	Diagnosis, remarks.
			Uric acid.	Urea N.	Creatinine.			
	yrs.		*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*	*mm. Hg*	*mg. per 100 cc.*	
J. G.	67	M.	5.7	21.7	1.4	150/80	0.2	Duodenal ulcer.
E. L.	16	F.	4.2	18.4	1.1	160/125	0.4	Mixed nephritis.
M. G.	16	"	4.0	12.0	1.0	148/74	0.2	Acute "
S. S.	54	"	4.4	12.8	1.1	135/90	0.3	Chronic colitis, chronic cholecystitis.
D. B.	62	M.	4.6	15.4	1.1	248/118	0.6	Chronic interstitial nephritis.
W. S.	62	"	4.7	21.0	1.6	190/130	0.3	Arteriosclerosis, cerebral double hemiplegia, dementia.
						190/130	0.4	Second determination, 6 days later.
A. S.	62	M.				150/80	0.4 0.4	Neurosyphilis, chronic progressive vascular nephritis; duplicate determinations.
W. P.	68	M.	3.3	14.7	3.6	158/76	0.2	Enlargement of prostate, hypertrophy, diverticula of bladder, chronic cystitis.
Average							0.33	

blood. Hence the amount found multiplied by 25 gives the amount of methylguanidine in 100 cc. of blood.

The results of a series of recoveries of methylguanidine added to human blood are recorded in Table I.

The color produced by the blood residue itself corresponds

to 0.2 mg. of methylguanidine per 100 cc. of blood. When the correction factor of 0.02 is applied per 10 cc. of blood (Column 5) the average per cent of recovery (Column 6) is 75.5 per cent.

Many control samples of blood were carried through the method side by side with samples having added methylguanidine. The color developed by quantities of methylguanidine less than 0.01 mg. is very slight. Due to this limitation in the method the best that can be said is that the control bloods contained less than 0.2 mg. of "guanidine" per 100 cc. of blood. The amount present may be actually 0.

A series of normal and hypertensive blood samples was analyzed with the technique described. The data are recorded in Tables II and III. There appears to be a slight but definite increase in the color reaction displayed by some hypertensive blood samples. It cannot be stated whether or not such increase is actually due to a guanidine base. These findings are in confirmation of those of Major and Weber (1927) and Major (1929) who found in cases of hypertension a content between 0.2 and 0.5 mg. of blood "guanidine" per 100 cc. of blood, while in cases of nephritis with considerable nitrogen retention the figures ranged from 0.3 to 2.3 mg.

SUMMARY.

1. A study has been made of the colorimetric reaction of certain guanidine bases with alkaline oxidized nitroprusside and ferricyanide.

2. Weber's method (1927) for the estimation of guanidine bases in blood has been modified to exclude interference by creatine.

3. The modified method has been applied to a few normal and hypertensive blood samples.

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THE PREPARATION OF THE CRYSTALLINE FOLLICULAR OVARIAN HORMONE: THEELIN.*

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Theelin: the Crystalline Follicular Ovarian Hormone.

In view of the many names suggested by investigators who have worked with the ovarian hormone which produces estrus in the ovariectomized female, it has become somewhat difficult to select a name for the pure crystalline product. Many of the names now in use are of value because of the associated ideas which the names suggest. Such names as folliculin, estrin, and progynon appear to be satisfactory in many respects but cannot be accepted because they have already been utilized as trade names by pharmaceutical houses for extracts containing only the partially purified hormone. The isolation of the crystalline hormone seems to justify the selection of a new name which should be reserved for the crystalline material and which in reality becomes the name of a new and probably hitherto unknown chemical substance. Accordingly, we suggest the term "theelin."[1] This word is derived from the Greek word "theelus" used both adjectively and nominally to signify "female." It is believed that this word is satisfactory from the standpoints of derivation and brevity.

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[1] The term theelin was adopted after a discussion of possible names with Dr. A. M. Schwitalla of St. Louis University and Dr. Nicholas M. Leech of the American Medical Association Laboratories. Owing to the possibility that solutions of the crystalline hormone may be used clinically, this name was submitted to the Council on Pharmacy and Chemistry, American Medical Association, which has recognized and approved it.

Preparation of Extracts.

Since our announcement at the meeting of The Thirteenth International Physiological Congress (1929) of the isolation of the crystalline hormone, both Butenandt (1929) and Laqueur (Dingemanse, de Jongh, Kober, and Laqueur, 1930) have reported their success in obtaining crystals. Since neither goes into details of the preparation, it is impossible to discuss their work. Marrian (1929) and Wieland, Straub, and Dorfmüller (1929) have reported procedures for the preparation of highly purified extracts of urine. In some respects the process used by Marrian is similar to the one that we have employed.

Importance of Acidification before Extraction.

Though Zondek and Aschheim (1928) speak of acidifying the urine with acetic acid before extracting the hormone with ether, they do not state the reasons for this step. As reported in an earlier paper (Doisy, Veler, and Thayer, 1930), we have found that the extraction of the hormone with olive oil from alkaline urine is far from complete. This statement is more or less true for the other solvents that we have used. Even with purified preparations the extraction from an acidic solution is more complete than from an alkaline solution. Provisionally, we have interpreted this as a salt formation between the alkali and a very weakly dissociated hydrogen of the hormone.

In the first runs of the chloroform apparatus we used urine that had been siphoned from the natural precipitates but the pH of which was left unchanged. Yields of 300 to 400 units per liter were obtained. We have found that after acidification of the urine the yield was increased six- to sevenfold. In the later runs the siphoned urine specimens were acidified to Congo red, left a week or more, and put under reduced pressure to remove the dissolved carbon dioxide which might separate and "air log" the extraction tube.

Quantity Production Methods.

Continuous Liquid Extraction: Chloroform.—Early in our work with the urine of pregnant women, it became apparent that the discovery of Aschheim and Zondek (1927) had led to a cheap, abundant source of the hormone. It was necessary to find suit-

able procedures for the extraction of huge quantities of the urine. After obtaining data upon the distribution between various possible solvents and urine, we decided upon the use of chloroform for the continuous liquid extractors. Our choice was based upon several factors: (1) favorable distribution of hormone between urine and chloroform, (2) the low solubility of inert substances in chloroform, (3) large difference in specific gravity between urine and chloroform, (4) little tendency of the chloroform and urine to form emulsions, (5) minimal fire hazard, and (6) moderate cost of reagent, as the loss by solubility in urine is small.

After constructing various forms of continuous liquid extractors and gaining information about their strong and weak points from actual laboratory trial, the apparatus described below was evolved. It gave satisfactory results but if we were to reconstruct it we would probably change to the counter-current principle which was used with the butyl alcohol apparatus.

In this extractor there is a circulation of the solvent and a continuous flow of the liquid being extracted. This enables us to extract large quantities at one run. The chloroform is boiled in the flask, A, (Fig. 1) and passes to the condenser, B (not shown), into the upper end of which the urine is introduced. The two pass through the extraction tube, C, to the separator, D, the chloroform traveling the faster. This tube is in the form of a zigzag of eight 32 inch lengths, having a slope of $2\frac{1}{4}$ inches to the foot. The separator is a 15 inch cylinder at the bottom of the apparatus. From here the chloroform passes through its return tube, E, which reaches to the bottom of the separator, back to the boiler. In the horizontal part of this tube we have an arrangement that has been found useful in cleaning the apparatus. The urine passes from the top of the separator through its overflow tube, F. This tube reaches to near the top of the apparatus and is made up of two telescoped tubes connected by rubber (shown in the insert). This gives it the adjustable length needed in regulating the apparatus. To obtain the return of the chloroform and the overflow of the urine, the pressures of these must be equal and the tube, F, is adjusted so that the height of the chloroform return above the separator interphase multiplied by its density will be equal to the urine overflow height multiplied by its density. Half inch Pyrex tubing was used from the flask to the extraction tube, quarter inch

being used elsewhere. The rate of extraction was about 8 gallons per 24 hours. Due to the simplicity of the apparatus, the watchman tended it at night, thus allowing it to be run as long as 7 days without a stop. The urine which had passed through

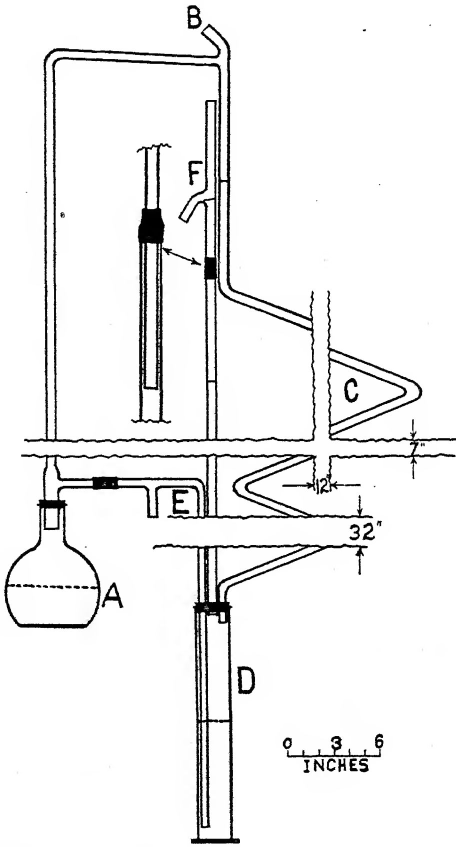

FIG. 1. Continuous liquid extraction apparatus for the use of chloroform as solvent. A, flask; B, condenser; C, extraction tube; D, separator, E, solvent return tube; F, overflow tube.

was collected and again extracted. After each run the chloroform solution was filtered, distilled, and the residue taken up in ethyl alcohol. The alcoholic solutions were assayed and then purified as described later.

Table I gives the data of typical runs. Following the last chloroform extraction, a sample of the urine was extracted in a separatory funnel with several portions of butyl alcohol and this extract tested for the hormone. The values obtained are given in Table I as "Remainder." Butyl alcohol was used, as we have obtained the largest yields from urine with this solvent. Three extractions in a separatory funnel with one-fourth as much butyl alcohol remove the hormone so completely that the fourth extraction contains less than 1 per cent of the total.

Continuous Liquid Extractor Based on Counter-Current Principle: Butyl Alcohol.—As our work progressed, the need for the hormone became so pressing that the capacity of the chloroform extractor became inadequate. Since we were confronted with the necessity of building more apparatus for extraction, improvements were considered. The counter-current principle was adopted to secure greater efficiency. We had also discovered that butyl alcohol is a better solvent than chloroform for the hormone and, though we realize that the loss of this solvent through its solubility might be rather large, we decided to build the new apparatus for butyl alcohol. At the same time some flexibility was included so that other solvents of approximately the same specific gravity, such as benzene, could be used if desired.

TABLE I.

Extraction with Chloroform in Continuous Extractor.

The yield is measured in rat units.

	Extraction No.	Yield.	Yield per gal.	Per cent of total.	Per cent efficiency.
Preparation AB; 50 gal. acidified urine.	1	416,000	8,320	78.7	78.7
	2	83,300	1,666	15.8	74.8
	3	10,000	200	1.9	34.5
	Remainder.	18,900		3.6	
		528,200			
Preparation AC; 50 gal. acidified urine.	1	500,000	10,000	72.1	72.1
	2	104,200	2,084	15.0	53.8
	Remainder.	89,100		12.9	
		693,300			

This butyl alcohol extractor (Fig. 2) required the addition of an upper separator (the Kjeldahl flask) and a rearrangement of the

tubes. The overflow tube, F, for the extracted urine must be lower than the solvent return, E, and reach to the bottom of the lower separator, D. The other tubes, the extraction tube proper and the butyl alcohol inlet, connect with the top of this separator. The solvent return is from the upper separator into which the urine is run.

The extraction tube, C, which is made of $\frac{3}{4}$ inch baffled tubing, connects the two separators. The solvent return is 8 feet from

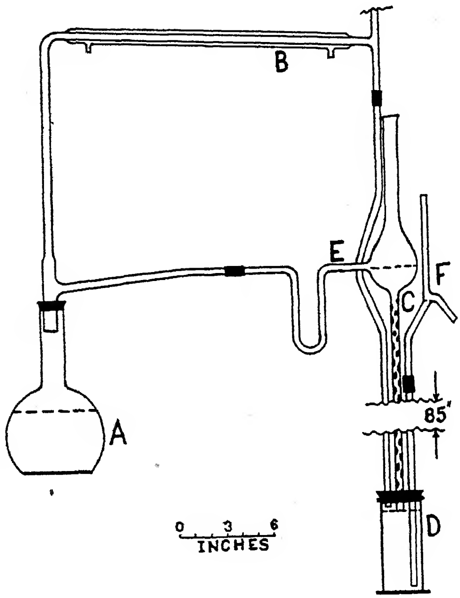

FIG. 2. Continuous liquid extraction apparatus for the use of solvents lighter than water (butyl alcohol). A, flask; B, condenser; C, extraction tube; D, separator; E, solvent return tube; F, overflow tube.

the lower separator and the urine overflow is 6 to 8 inches lower than this. The rate of this extractor is from 25 to 30 gallons of urine per 24 hours.

In Table II we are giving data on the extraction of the hormone from the mixed acidified urine specimens of pregnant women. On Preparations AE and AD a second passage through the extractor gave a small additional quantity of the active substance. It would seem that the yield does not justify the time required for the second extraction.

The potency of the butyl alcohol extracts was determined by distilling a suitable aliquot, dissolving the residue in olive oil, and applying our routine procedure of assay in which the volume injected is divided into 3 equal parts.

TABLE II.

Extraction of Mixed Urine Specimens with Butyl Alcohol.

Preparation.	Volume of acidified urine.	Rat units per liter.	
		Extraction 1.	Extraction 2.
	liters		
AE	190	4210	177
AD	108	3180	315
AJ	850	4240	

TABLE III.

Leaching of Residue of Butyl Alcohol Extract with Benzene.

Preparation No.	Soluble in benzene.	Insoluble in benzene.
	rat units	*rat units*
132	25,000	2,870
136	45,000	1,180
142	12,600	1,570
144	27,000	1,960
147	139,000	4,560
A 150	95,000*	
151	95,000	1,980
154	136,000	1,960
Total................	479,600	16,080

Average loss is 3.2 per cent.
* Omitted from calculation of percentage loss.

Purification of Extracts.

1000 cc. volumes of the butyl alcohol extracts containing approximately 100 rat units per 1 cc. are distilled, a boiling water bath and water pump being used to remove the butyl alcohol. The residue is extracted by heating and shaking with 400 cc. of benzene; upon cooling, the benzene can readily be poured off from the tarry mass in the bottom of the flask. The leaching is repeated twice with 200 cc. of benzene. These extracts are combined and

chilled; the small amount of precipitate is added to the other benzene-insoluble residue. In Table III we have given data illustrating the efficiency of this step of the purification. The average loss of the hormone of seven preparations is about 3 per cent, whereas our estimate of the removal of inert substances varies from 25 to 50 per cent.

Several alternative procedures have been used for the purification of the benzene solution, the important objective being the attainment of an alkaline aqueous solution of the hormone. In our earliest work with urine it seemed that losses were incurred

TABLE IV.

Extraction of Butyl Alcohol-Petroleum Ether Solution with Dilute NaOH.

Preparation No.	Final volume of NaOH solution.	Volume of butyl alcohol-petroleum ether solution.	Alkaline solution.	Petroleum ether solution.
	cc.	*cc.*	*rat units*	*rat units*
134 c	616	500	246,000	10,000
136 c	760	375	15,200	12,500
142 c	1265	1000	25,300	1,100
151 c	1388	1000	80,000	5,330
154		1000	134,000*	2,230
A 142 c	344	300	43,000	1,000
A 147	490	300	122,500	17,500
A 156	771	200	493,000	3,600
A 158	750	300	96,000	1,200
Total			1,255,000	54,460

Average loss is 4.2 per cent.

* By difference.

by boiling with sodium hydroxide; subsequently, we have sought means of transferring the hormone to solution in aqueous alkali without heat. In this we have succeeded (petroleum ether-butyl alcohol procedure) but as our work progressed the fear of loss from hot alkali diminished and we began extracting the tar with warm 0.5 N NaOH and finally extracted with boiling alkali. All of these modifications seem to work satisfactorily, the hormone dissolving rather readily in 0.3 N alkali.

The benzene solution is distilled with a water pump to complete the removal of the benzene. The tar is transferred to a separatory

funnel with 50 cc. of butyl alcohol and 1000 cc. of petroleum ether added. The extraction is effected by shaking with 400 cc. of 0.5 N NaOH, then 400 cc., 200 cc., and 200 cc. of 0.25 N NaOH. The solution is chilled and poured from the tar which sticks to the bottom and sides of the flask. Data obtained by this distribution are shown in the upper part of Table IV.

Though the data are not complete enough for detailed discussion, we have accomplished the preparation of this alkaline solution by transferring the residue from the benzene solution to a separatory funnel with a small volume of butyl alcohol. 300 cc. of 0.5 N NaOH heated to 75° are added and the mixture stirred with a mechanical stirrer for 20 minutes. The tar rises to the top and the aqueous solution can be tapped off. This extraction is generally repeated four times. The aqueous solutions are combined, the alkali partially neutralized, and the solution chilled and then poured off from the tar which separates.

In our earlier preparations the alkaline solution was extracted with ethyl ether, the ether distilled, and the residue dissolved in a small volume of butyl alcohol. 20 volumes of petroleum ether were added and this solution was extracted with dilute NaOH. The distribution of the hormone between the residual butyl alcohol-petroleum ether layer and the alkaline solution is shown by the data of the lower part of Table IV. In the experiments showing the less favorable distribution we suspect that the concentration of the alkali was insufficient for thorough extraction, since in our later work in which stronger alkali was used the loss of the hormone was less. A large proportion of the inert substances was eliminated at this stage.

Recently we have found that the second extraction of the butyl alcohol-petroleum ether solution is unnecessary. After the extraction of the first alkaline solution with ethyl ether the ether is distilled and the residue is steam-distilled as long as volatile products are removed. The condensed steam is removed by distillation, leaving a small quantity (1 to 3 gm.) of a dark tarry material which is extracted with 25 cc. of hot 0.25 N NaOH. Upon cooling the mass sticks to the flask and the alkaline solution can be poured off. The leaching is repeated four or five times and the combined extracts filtered through a Jena glass filter (porosity <7). This alkaline solution contains nearly all of the hormone, the

residual tar possessing very little activity. The data of Table V are illustrative. The average loss in this step is less than 1 per cent of the total.

The alkaline solution is extracted with one-fourth of its volume of ethyl ether 6 times, and the combined ether solutions washed

TABLE V.

Leaching with Dilute NaOH.

Preparation No.	Hot 0.25 N NaOH. In alkaline solution.	Hot 0.25 N NaOH. Undissolved by NaOH.
	rat units	*rat units*
A 1561	235,000	1600
A 158 a	96,000	1400
162	177,000	1000
B 165	128,000	860
A 166	87,100	1005
Total..................	723,100	5865

Average loss is <1 per cent.

TABLE VI.

Leaching with Dilute NaOH.

Preparation No.	Cold 0.25 N NaOH. In alkaline solution.	Cold 0.25 N NaOH. Undissolved by alkali.
	rat units	*rat units*
D 142	50,000	1,040
D 156	184,000	13,000
D 158	53,000	2,333
D 159	191,000	20,000
D 162	58,000	2,360
D 165	57,000	20,000
Total................	593,000	58,733

Average loss is 9 per cent.

once with 20 cc. of 0.1 N $NaHCO_3$, once with 20 cc. of 0.1 N HCl, and once with water. The ether is distilled and the residue dried by vacuum distillation. This residue is then extracted with 20 cc. of cold (25°) 0.25 N NaOH. The alkaline solution is poured off, 2 cc. of ethyl alcohol are added to the residue, and the alcohol is

distilled. The extraction with alkali is repeated in this way four or five times and the combined aqueous solutions filtered. While the use of the alcohol to expose a new surface to the extraction with alkali may not be necessary, our experience indicates that it is desirable. The data of Table VI give the reader an idea of the efficiency of this extraction.

The alkaline solution is extracted with sufficient ethyl ether so that approximately 10 cc. of ether per 100 cc. of aqueous solution will remain undissolved. The aqueous solution is tapped off and this ether solution worked back into an earlier stage of a subsequent preparation. The extraction of the aqueous solution with one-fourth of its volume of ether is carried out five or six times and the combined ether extracts washed as in the preceding step. The ether is distilled, and a few cc. of ethyl alcohol added, distilled to dryness. The residue, which is generally a stiff, light yellow oil, in which crystals may appear on standing, is transferred with 3 cc. of ethyl alcohol to a 50 cc. centrifuge tube, 9 cc. of hot water added, and the solution seeded. Upon cooling slowly a semicrystalline mass usually forms. The tube is chilled to $-10°$ and then filtered through a micro Jena glass filter (porosity <7). The precipitate is dissolved with hot alcohol and sucked through into the centrifuge tube. The alcohol is evaporated and the residue dissolved in an appropriate volume of hot butyl alcohol (0.25 to 0.75 cc.). Upon seeding and cooling, crystals generally form. The addition of 2 to 5 volumes of petroleum ether decreases the solubility of the hormone without precipitating inert substances. After chilling to $-10°$, the crystals are filtered off; if they are not almost white, a repetition of the butyl alcohol crystallization may be desirable. If almost white, recrystallization from 10 cc. of hot 20 per cent alcohol is satisfactory. Recrystallization should be repeated until the melting point is about 243° (uncorrected). Both Butenandt and Laqueur report melting points of 240° but our pure crystals from several preparations melted with decomposition when heated *slowly* at temperatures ranging from 243–243.5° (uncorrected).

An actual example of the preparation of the crystals from the crude butyl alcohol extract by the procedure just described is given below.

Preparation 156.—4 liters of butyl alcohol extract were distilled, leached with benzene, and transferred to alkaline aqueous solution by the butyl alcohol-petroleum ether procedure. The alkaline solution was extracted with ether, the ether distilled, and the residue steam-distilled. This residue was dissolved in 10 cc. of butyl alcohol and 200 cc. of petroleum ether were added; the solution was extracted four times with 100 cc. of dilute NaOH. 15 cc. of butyl alcohol were added to the petroleum ether to effect the solution of some tarry material which had separated. The solution was extracted twice with dilute NaOH and the alkaline aqueous solutions were combined.

Volume 771 cc. — Left in petroleum ether (3600 rat units)

493,000 rat units

Extracted with ethyl ether; ether distilled; residue leached with cold NaOH

Solution extracted with ethyl ether; distilled — Insoluble residue, 1600 rat units

By crystallization from aqueous alcohol, butyl alcohol, and petroleum ether 32 mg. of crystals isolated

Cruder fractions worked back into purification of another sample

DISCUSSION.

A few points in the preparation of the hormone might be clarified or improved by further work. However, this might involve another year of study which, judging from the requests for information, is undesirable. On the other hand, our experience indicates that the procedure outlined is satisfactory in the two most important particulars; *i.e.*, the loss of the hormone is small, and a crystalline product is almost invariably attained. Of the last fifteen preparations attempted, all were obtained in crystalline form.

Though the method may appear complicated, what else could one expect from an extraction and purification procedure designed to recover a solute in crystalline form when it exists in a concentration of less than 1 part in 1,000,000? We may add that the procedure is not so difficult to follow, since most of it is now being executed successfully by a young man who has had no previous training in chemistry.

The essential steps depend upon the solubility of the hormone in butyl alcohol, benzene, ethyl ether, and dilute sodium hydroxide. The most important purification seems to depend upon the separation of the substances into alkali-soluble and alkali-insoluble, coupled with the fact that of the alkali-soluble very few are extracted with ethyl ether from the alkaline solution. Repetition of the alkaline extraction at room temperature is important.

In the event that one fails to obtain crystals by the procedure outlined, it is only necessary to dump the various purified fractions back into a flask, distil to dryness, and repeat the leaching with alkali at room temperature. Ether extraction and the crystallization procedure produce the crystals. Occasionally we have found it necessary to resort to this additional purification.

We are aware that the extraction of an alkaline solution with ethyl ether does not give a quantitative recovery of the hormone. However, the per cent lost and purity of the ether extract depend upon the alkalinity of the solution. We prefer to extract only a part of the hormone in order that the purification may be more efficient. The alkaline solutions generally have a pH between 9 and 10 during the ether extraction. We save the alkaline solutions until several gallons have accumulated, then add acid until the pH is about 8.0 (acid to phenolphthalein but alkaline to phenol red), and extract with butyl alcohol in the continuous liquid extractor. After this extraction the aqueous solution contains about 1 rat unit per cc. This butyl alcohol solution is much less contaminated than the butyl alcohol extract of urine and consequently may be more readily purified. We have recently recovered over 1,000,000 units from 60 liters of the alkaline solution.

A résumé of the steps of the procedure of the preparation of the crystalline hormone, theelin, may be of value to the reader. The losses in the various steps are taken from Tables I to VI given in this paper.

1. Urine; butyl alcohol extraction in continuous liquid extractor
2. Butyl alcohol distilled; residue leached with benzene

2a. Solution

2b. Insoluble residue discarded; 3.2 per cent loss

3. Distilled and residue transferred to solution in dilute NaOH

3a. Alkaline solution

3b. Insoluble tar discarded; 4.2 per cent loss

4. Extracted with ethyl ether

5. Ether distilled; residue steam-distilled

6. Residue leached with hot 0.25 N NaOH

6a. Solution

6b. Residue discarded; 1 per cent loss

7. Extracted with ethyl ether; ether distilled

8. Residue leached with cold 0.25 N NaOH

8a. Solution extracted with ether; ether distilled

8b. Insoluble residue discarded; 9 per cent loss

9. Residue crystallized from hot 25 per cent ethyl alcohol or butyl alcohol

SUMMARY.

A quantity production procedure for the preparation of theelin is described. Essentially the procedure depends upon the fact that theelin, which behaves as a very weak acid, may be extracted from organic solvents with dilute NaOH solutions, and then in turn may be extracted from the alkaline solution by certain organic solvents.

We wish to add that the progress of our work has been facilitated by the assistance of Mr. Louis Levin to whom we express our appreciation.

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CRYSTALLOGRAPHIC DESCRIPTION OF THEELIN.

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The following optical properties were determined upon material supplied by Dr. Doisy, which had been recrystallized twenty times, the final recrystallization being from 20 per cent alcohol.[1] The crystals are monoclinic with a distinct tabular development

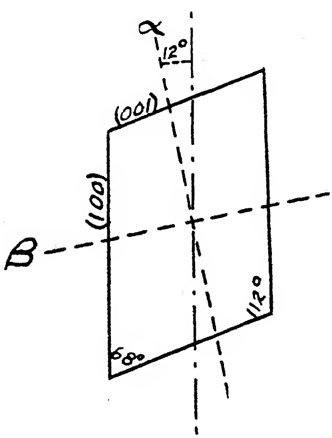

FIG. 1.

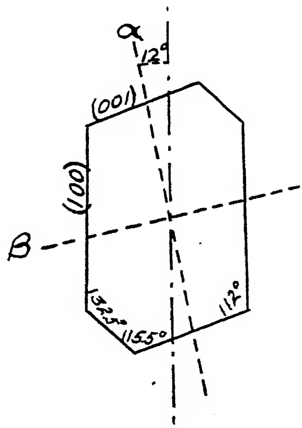

FIG. 2.

FIGS. 1 AND 2. Diagram illustrating optical properties of theelin.

parallel to the side pinacoid and have a rhomboid outline (Fig. 1) with included angles of 112° and 68°. In many cases the acute angle of the rhomboid is truncated by a small crystal face giving a six-sided outline (Fig. 2) with included angles of 112°, 115.5°, and 132.5°. No crystals of this character are shown in the photo-

[1] Assay = 4000 rat units per mg. Melting point with decomposition = 243.5° (uncorrected).

graphs (see Figs. 1 and 2, Doisy, Veler, and Thayer[2]). This variation in outline is superficial and represents only variations in the conditions under which precipitation took place.

The crystals are optically negative; the indices of refraction are $\alpha = 1.520$, $\beta = 1.642$, and $\gamma = 1.692$ (all $\pm$ 0.003), and $2V = 50$–$55°$. The obtuse bisectrix is perpendicular to the side pinacoid and therefore may be observed on all the rhomboid crystals. The extinction angle with the longer edge of these crystals is 12° (in the direction of the obtuse angle). α is measured parallel to this direction and β perpendicular to it. The crystals possess a pronounced basal cleavage giving sections nearly perpendicular to the acute bisectrix upon which β and γ may be measured. Although the crystals are very small they are extremely well developed and adapt themselves readily to micro optical determinations.

[2] Doisy, E. A., Veler, C. D., and Thayer, S., *J. Biol. Chem.*, **86**, 505 (1930).

CONFIGURATIONAL RELATIONSHIPS OF PHENYLATED CARBINOLS.

By P. A. LEVENE and P. G. STEVENS.

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(Received for publication, April 8, 1930.)

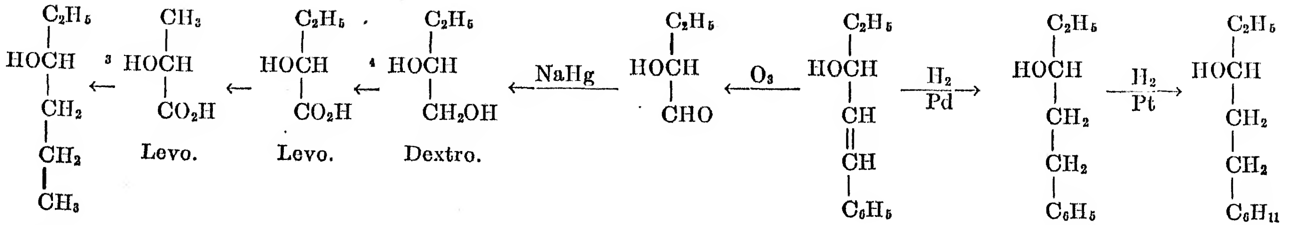
All the carbinols which have been directly correlated to lactic acid have been strictly aliphatic. A number of carbinols with phenyl groups has been studied but none correlated. Hewitt and Kenyon[1] pointed out that the phenylated carbinols possess much higher activities than the corresponding aliphatic ones of the same number of carbon atoms, and that the enhancement apparently increases as the phenyl group approaches the asymmetric carbon atom. But they made no direct correlations and must have assumed that the phenyl group does not cause an inversion of the sign of rotation.

This paper deals with the correlation of a number of carbinols containing a phenyl group at different distances from the asymmetric carbon atom. The first studied was ethyl-β-phenethyl carbinol II which was correlated to lactic acid in the manner shown in the accompanying diagram.

The results of these correlations show that levo-ethyl-β-phenethyl carbinol II and levo-ethyl-β-cyclohexethyl carbinol III are related to levo-ethylpropyl carbinol, that a phenyl group in the β position to the asymmetric carbon atom causes no inversion of the sign of rotation, but rather an enhancement, and that a cyclohexyl group likewise in the β position is roughly equivalent to a normal hexyl group. Further, the presence of a double bond in the α, β position as in I causes an inversion of sign just as in all other similarly unsaturated carbinols.[2]

[1] Hewitt, L. F., and Kenyon, J., *J. Chem. Soc.*, **127**. 1094 (1925).

[2] Levene, P. A., and Haller, H. L., *J. Biol. Chem.*, **83**, 579 (1929).

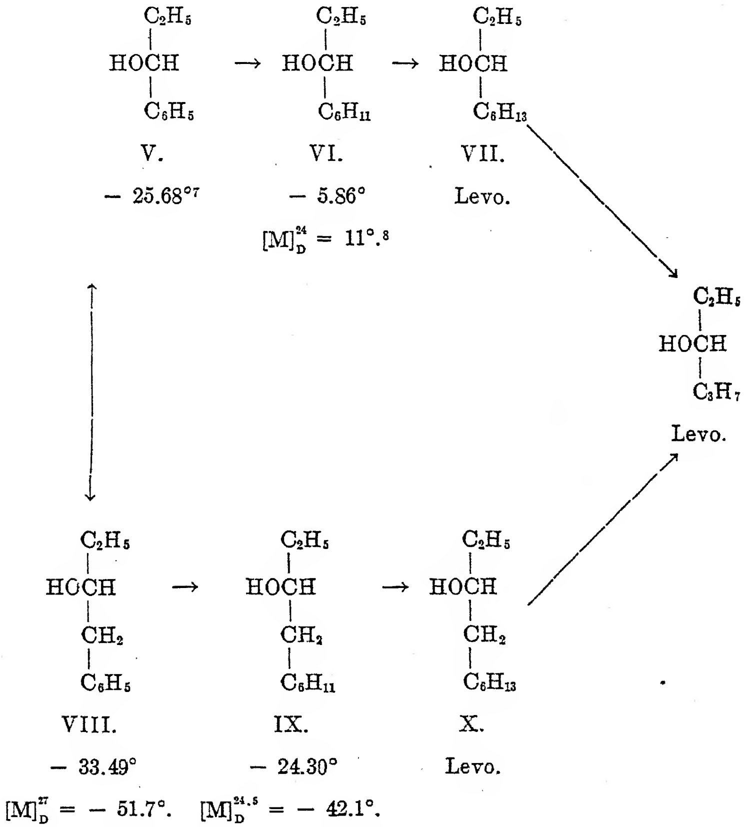


Levo. Levo. Levo. Dextro. I. $+3.3°$ II. $-12.95°$ III. $-3.89°$

$[M]_D^{24} = 10°$.[5,6]

[3] Levene, P. A., and Haller, H. L., *J. Biol. Chem.*, **76**, 415 (1928).
[4] Levene, P. A., and Haller, H. L., *J. Biol. Chem.*, **74**, 343 (1927).
[5] Calculated approximately from the maximum value of Hewitt and Kenyon's ethyl-β-phenethyl carbinol.
[6] Hewitt and Kenyon found $[M]_D^{20}$ for ethyloctyl carbinol to be 10.7.

The fact that a cyclohexyl group is about equivalent to a normal hexyl group made it possible to correlate carbinols with the phenyl group directly attached to and once removed from the asymmetric carbon atom with a reasonable degree of probability. The reduction of the phenyl group in each case resulted in a decrease in activity but no change of sign.



The correlations VI to VII and IX to X rest upon two considerations: First, the fact that direct correlation showed that a cyclo-

[7] This material was prepared by Levene and Mikeska (Levene, P. A., and Mikeska, L. A., *J. Biol. Chem.*, 70, 355 (1926)).

[8] Calculated approximately from Pickard and Kenyon's data (Pickard, R. H., and Kenyon, J., *J. Chem. Soc.*, 99, 45 (1911)). Pickard and Kenyon give $[M]_D^{20}$ = 10.6 for ethylhexyl carbinol.

hexyl group in the β position is about equivalent to the normal hexyl group, no inversion of sign taking place, and second, that the cyclohexyl group is a saturated radical in its chemical nature and resembles a straight chained group rather than a branched chain or a polar radical (with the possible exception of the $C_6H_{11}CH_2$ group). Thus it merely becomes the heavier group, and by the rule of Levene and Haller[9] should be related to other carbinols having the heavier group in the same position. But these correlations must be considered tentative until the relation between cyclohexyl and normal hexyl groups has been established by direct evidence.

The effect on the rotation of the distance of the phenyl group from the asymmetric carbon atom as yet cannot be summed up in the form of one simple rule. Hewitt and Kenyon report the following molecular rotations for the methyl series.

A	
$CH_3CH(OH)Ph$	50.9
$CH_3CH(OH)CH_2Ph$	36.1
$CH_3CH(OH)CH_2CH_2Ph$	21.3

B		Δ (A − B)	Ratio $\frac{A}{B}$
$CH_3CH(OH)C_6H_{13\,(n)}$	12.7	38.2	4:1
$CH_3CH(OH)CH_2C_6H_{13\,(n)}$	12.9	23.2	3:1
$CH_3CH(OH)CH_2CH_2C_6H_{13\,(n)}$	13.7	7.6	1.5:1

Thus the authors conclude that the enhancing effect of the phenyl group drops with the increase in the distance.[10]

The state of affairs seems to be different in the ethyl series.

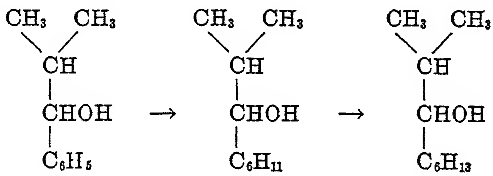
A		B	
$C_2H_5CH(OH)Ph$	35.2[1]	$C_2H_5CH(OH)C_6H_{13\,(n)}$	10.6[1]
$C_2H_5CH(OH)CH_2Ph$	51.7	$C_2H_5CH(OH)CH_2C_6H_{13\,(n)}$	10.6[11]
$C_2H_5CH(OH)CH_2CH_2Ph$	31.0[1]	$C_2H_5CH(OH)CH_2CH_2C_6H_{13\,(n)}$	10.7[1]

[9] Levene, P. A., and Haller, H. L., *J. Biol. Chem.*, **79**, 475 (1928).
[10] Compare Kuhn, W., *Ber. chem. Ges.*, **63**, 199 (1930).
[11] Pickard, R. H., and Kenyon, J., *J. Chem. Soc.*, **103**, 1933 (1913).

C		Δ (A−B)	Δ (A−C)	$\frac{A}{B}$	$\frac{A}{C}$
$C_2H_5CH(OH)C_6H_{11}$	11[8]	24.6	24.2	$\frac{3.3}{1}$	$\frac{3.2}{1}$
$C_2H_5CH(OH)CH_2C_6H_{11}$	42.1[12]	41.1	9.6	$\frac{4.9}{1}$	$\frac{1.25}{1}$
$C_2H_5CH(OH)CH_2CH_2C_6H_{11}$	10[5]	20.3	21	$\frac{2.9}{1}$	$\frac{3.1}{1}$

Hence the regularity observed in the methyl series seems to be lacking in the ethyl series. The difference may be real, but there is an equal probability that it is only apparent due to the fact that the values accepted by Hewitt and Kenyon as the maxima, are in reality the values for the substances only partially resolved.

In all the above cases, only one polar group is present; the phenyl group. A study of isopropylphenyl carbinol was of interest therefore because of the two different polar groups. Reduction here caused an inversion of sign as well as a decrease in activity.



XI. XII. XIII.

− 9.01°[7,13] + 2.75° Dextro.

Due to certain irregularities in the isopropyl series, the correlation of XII to XIII is tentative. Also, it is not possible as yet to cor-

[12] The irregularity of rotatory power here shown by these cyclohexyl carbinols may be related to the irregularity of dissociation constants exhibited by a similar set of acids.

	K at 25°*
$C_6H_{11}CO_2H$	1.34×10^{-5}
$C_6H_{11}CH_2CO_2H$	2.36×10^{-5}
$C_6H_{11}CH_2CH_2CO_2H$	1.34×10^{-5}

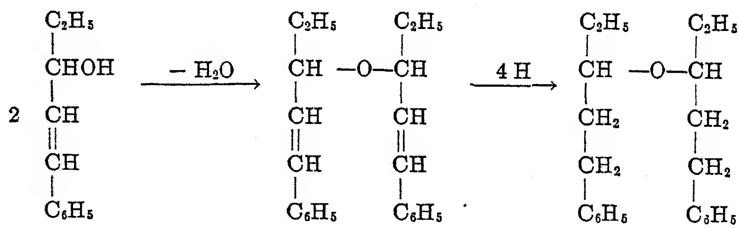
* Zelinsky, N., and Izgaryschew, N., *Chem. Zentr.*, 1, 532 (1909).

[13] Highest value found $\alpha_D^{23} = +20.23°$.

relate XIII with the normal secondary carbinols. A further investigation in this direction is now in progress.

During the reductions of these phenylated carbinols, considerable amounts of hydrocarbons were formed. Adams' platinum oxide catalyst was used with hydrogen at 30 pounds pressure in glacial acetic acid solution. It was found that in those cases where the phenyl group was directly attached to the asymmetric carbon atom, as much as two-thirds of the material was reduced to the corresponding hydrocarbon. Where the phenyl group was once removed, only about 8 per cent was reduced to the hydrocarbon and where twice removed, practically none.

Difficulty was experienced in isolating active ethylstyryl carbinol in a pure state, because the carbinol readily forms a dimolecular ether which possesses a high activity as compared with that of the carbinol. The unsaturated character of the ether was apparent from its absorption of bromine and hydrogen. The reduction was carried out with Adams' platinum oxide catalyst, colloidal palladium being without effect. The reduced ether displayed a greatly decreased activity.



Dextro in alcohol. — Levo in alcohol. — Levo in ether ?

Levo in ether. — Levo in ether.

EXPERIMENTAL.

Ethylstyryl Carbinol.

The carbinol was prepared from cinnamic aldehyde by the action of ethyl magnesium bromide.[14] It was converted into the acid phthalate in the usual way with pyridine and phthalic

[14] We are indebted to Mr. R. E. Marker for the preparation of this carbinol.

anhydride. This, after two recrystallizations from ether and ligroin, melted at 108–109°, resolidified, and remelted at 160° ± with decomposition.

Resolution of the Acid Phthalate.—The alkaloid used was cinchonidine, for neither brucine nor strychnine gave crystalline salts. From acetone the salt of one modification crystallized out splendidly. After several recrystallizations from 95 per cent alcohol, the salt was decomposed, and the crystalline phthalate melting at 83.5–85° was examined. In absolute alcohol it had the following activity.

$$[\alpha]_D^{23} = \frac{-0.52° \times 100}{1 \times 17.40} = -3.0°.$$

The activity of various samples ranged between this value and −2.3°. A sample which in absolute alcohol was −2.4° at 27° had the following rotation in absolute ether.

$$[\alpha]_D^{27} = \frac{-0.18° \times 100}{1 \times 16.5} = -1.1°.$$

As the more soluble cinchonidine salt did not crystallize, it was converted to the crystalline phthalate, which had the following rotation in absolute alcohol.

$$[\alpha]_D^{23} = \frac{+0.40° \times 100}{1 \times 16.80} = +2.4°.$$

The usual procedure was employed for the saponification of the phthalate. The carbinol, however, boiled so high that it was extracted with ether rather than steam-distilled. Purification of the carbinol by distillation proved difficult. It did not boil at all constantly. Moreover, there was a large viscous residue,—probably the dimolecular ether, as it turned out to be highly optically active. From the above dextro-phthalate, the following fractions at 1.0 mm. were obtained.

1st fraction, boiling point 97–108°, No. 35.
2nd " " " 108–111°, " 36.

Both were dextrorotatory in absolute alcohol.

$$\text{No. 35.}\quad [\alpha]_D^{24} = \frac{+\ 0.25° \times 100}{1 \times 19.36} = +\ 1.3°.$$

$$\text{No. 36.}\quad [\alpha]_D^{24} = \frac{+\ 0.25° \times 100}{1 \times 17.92} = +\ 1.4°.$$

Redistillation of No. 36 yielded the following fractions at 0.8 mm.

1st	fraction,	boiling point	up to 101°,	No. 37.
2nd	"	" "	101–105°,	" 38.
3rd	"	" "	105–115°, small amount only.	
4th	"	Residue.	No. 40.	

Both No. 37 and No. 38 were levorotatory in absolute ether.

$$\text{No. 38.}\quad [\alpha]_D^{25} = \frac{-\ 0.93° \times 100}{1 \times 16.54} = -\ 5.6°.$$

No. 38 was redistilled. The fraction which boiled at 0.6 mm. at 112–115° gave the following analysis.

4.505 mg. substance: 13.480 mg. CO_2 and 3.485 mg. H_2O.
$C_{11}H_{14}O$. Calculated. C 81.5, H 8.6.
Found. " 81.2, " 8.7.

In absolute ether it had the following rotation.

$$[\alpha]_D^{26} = \frac{-\ 0.88° \times 100}{1 \times 16.26} = -\ 5.4°.$$

The residue, No. 40, probably the ether, gave the following analysis.

3.210 mg. substance: 10.135 mg. CO_2 and 2.405 mg. H_2O.
$C_{22}H_{26}O$. Calculated. C 86.2, H 8.5.
Found. " 86.1, " 8.4.

In absolute ether it had a high rotation.

$$[\alpha]_D^{25} = \frac{-\ 11.76° \times 100}{1 \times 16.56} = -\ 71.0°.$$

In absolute alcohol it was also levorotatory.

The formation of the ether takes place on distilling and also on standing at room temperature. For this reason it was difficult to be certain of the amount of rotation of the carbinol. Thus No. 35 which was dextrorotatory in absolute alcohol, after several weeks, became strongly levorotatory.

$$[\alpha]_D^{22.5} = \frac{-5.40° \times 100}{1 \times 23.26} = -23.2°.$$

In absolute ether it was also much more strongly levorotatory than the pure carbinol.

$$[\alpha]_D^{24} = \frac{-4.00° \times 100}{1 \times 20.66} = -19.3°.$$

The same carbinol prepared from another lot of dextro-phthalate ($\alpha_D^{24} = +2.3°$) was very carefully fractionated. After several distillations, a fraction was obtained which boiled at 115–118°, mostly at 118° at 0.2 mm. (metal bath at 140–145°). This was colorless, practically odorless, and had the following composition.

3.740 mg. substance: 11.290 mg. CO_2 and 2.905 mg. H_2O.

$C_{11}H_{14}O$.	Calculated.	C 81.5, H 8.6.
	Found.	" 82.3, " 8.7.

In absolute alcohol it was dextrorotatory.

$$[\alpha]_D^{23} = \frac{+0.39° \times 100}{1 \times 18.52} = +2.1°.$$

In absolute ether it was levorotatory.

$$[\alpha]_D^{23} = \frac{-1.07° \times 100}{1 \times 15.48} = -6.9°.$$

Without solvent, 1 dm. tube, $\alpha_D^{23} = -3.30°$.

The levo-phthalate ($[\alpha]_D^{24} = -2.4°$) in the same way gave a carbinol that was dextrorotatory. The crude carbinol after drying but before distillation had the following composition.

3.835 mg. substance: 11.575 mg. CO_2 and 2.870 mg. H_2O.

$C_{11}H_{14}O$.	Calculated.	C 81.5, H 8.6.
	Found.	" 82.3, " 8.4.

In absolute ether it had the following rotation.

$$[\alpha]_D^{22} = \frac{+\ 1.82° \times 100}{1 \times 17.52} = +\ 10.4°. \quad \text{No. 48.}$$

Doubtless this activity here is too high for the pure carbinol due to contamination by the active ether.

Phenylurethane of Dextro-Ethylstyryl Carbinol.—To 2 gm. of dextro-carbinol No. 48 were added 1.5 gm. of phenylisocyanate. The mixture was gently warmed for 15 minutes on the steam bath. On cooling it set to a crystal cake, which was ground up and washed well with ligroin. After two recrystallizations from absolute alcohol, the colorless needles melted at 112.5–113° and analyzed as follows:

9.600 mg. substance: 0.426 cc. N_2 at 25° and 753 mm.
$C_{18}H_{19}O_2N$. Calculated. N 5.0 Dumas.
Found. " 5.1.

The urethane had the following rotations.

$$\text{In absolute alcohol } [\alpha]_D^{26.5} = \frac{+\ 2.62° \times 100}{1 \times 2.24} = +\ 117.0°.$$

$$\text{In chloroform } [\alpha]_D^{25} = \frac{+\ 17.47° \times 100}{1 \times 15.04} = +\ 116.1°.$$

Dextro-Butylene Glycol.

Ozonization of Dextro-Ethylstyryl Carbinol.—11.4 gm. of carbinol No. 48, dissolved in 30 cc. of pure glacial acetic acid, were ozonized at 0° until the solution no longer added bromine. The decomposition of the ozonide was carried out according to Helferich[15] in the presence of zinc. The zinc was filtered off and carefully washed with ether and glacial acetic acid. The filtrate was concentrated *in vacuo*, neutralized with sodium hydroxide; the zinc hydroxide was filtered off and washed well with alcohol and water. This filtrate, consisting of about 350 cc. of 50 per cent alcohol, was treated with sodium amalgam until the solution no longer reduced Fehling's solution. The mixture was then filtered, neutralized with 10 per cent sulfuric acid and concen-

[15] Helferich, B., *Ber. chem. Ges.*, **52**, 1123 (1919).

trated under reduced pressure. Upon addition of acetone, all the sodium acetate was precipitated. Reconcentration and further addition of acetone produced no further precipitate. The solution was then reconcentrated, water added, and filtered. Again the filtrate was evaporated under reduced pressure. The residual oil was taken up in ether, dried over sodium sulfate, and the ether evaporated. The remaining liquid, after drying at 70° at 0.6 mm. weighed 2.8 gm., a yield of about 25 per cent.

Dextro-Butylene Glycol Di-(Phenylurethane).—The above oil was treated with an excess of phenylisocyanate. The mixture warmed energetically by itself and was further heated for 2 to 3 hours on the steam bath. On chilling, only a paste was obtained. After successive extractions with ligroin and later with mixtures of ligroin and ether, most of the benzyl alcohol urethane was removed, and the paste then solidified. After recrystallizing twice from benzene and ligroin, and thrice from dilute alcohol, the fine needles melted at 124–125°.

The mixed melting point with a sample of dextro-butylene glycol di-(phenylurethane) (melting point 124–125°, $[\alpha]_D^{20} = +23.7°$)[16] was 124°–125°. The di-urethane had the following rotation in absolute alcohol.

$$[\alpha]_D^{21.5} = \frac{+1.04° \times 100}{1 \times 4.46} = +23.3°.$$

It analyzed as follows:

4.250 mg. substance: 10.450 mg. CO_2 and 2.395 mg. H_2O.
9.350 " " : 0.7644 " N_2 (Van Slyke).

$C_{18}H_{20}O_4N_2$.	Calculated.	C 65.9, H 6.1, N 8.5.
	Found.	" 66.6, " 6.3, " 8.2.

Levo-Ethyl-β-Phenethyl Carbinol.

Reduction of Dextro-Ethylstyryl Carbinol.—10 gm. of dextro carbinol No. 48, dissolved in ether, were shaken with aqueous colloidal palladium and hydrogen. After about 6 hours, when the absorption seemed complete, the ether was separated, dried over sodium sulfate, and evaporated. The residual oil was distilled. The fraction distilling slowly at 87–88° at 0.4 mm., solidified in

[16] Levene, P. A., and Haller, H. L., *J. Biol. Chem.*, **74**, 349 (1927).

the receiver to long white spears melting at 38–41°, and analyzed as follows:

4.395 mg. substance: 13.030 mg. CO_2 and 3.830 mg. H_2O.
$C_{11}H_{16}O$. Calculated. C 80.5, H 9.8.
Found. " 80.8, " 9.8.

In absolute ether it had the following rotation.

$$[\alpha]_D^{24} = \frac{-3.14° \times 100}{1 \times 16.72} = -18.8°.$$

Without solvent, 1 dm. tube, $\alpha_D^{25.5} = -12.63°$.

Another sample, from a slightly more active phthalate ($[\alpha]_D^{23} = -2.9°$) was carefully fractionated. The fraction boiling at 103–107° (rapidly distilled) at 0.1 mm.[17] solidifying as before in the receiver, was considered pure and had the following rotations.

Without solvent, 1 dm. tube, $\alpha_D^{25} = -12.95°$, $[\alpha]_D^{25} = -13.3°$, $[M]_D^{25} = -21.8°$.[18]

$$\text{In absolute alcohol } [\alpha]_D^{25} = \frac{-2.63° \times 100}{1 \times 14.74} = -17.8°.$$

$$\text{In absolute ether } [\alpha]_D^{25.5} = \frac{-2.93° \times 100}{1 \times 16.48} = -17.7°.$$

α-Naphthylurethane of Levo-Ethyl-β-Phenethyl Carbinol.—To 1 gm. of the saturated carbinol ($[\alpha]_D^{24} = -18.0°$ in ether) were added 1.5 gm. of α-naphthyl isocyanate. The mixture crystallized after 1 hour on the steam bath. After chilling, the crystals were washed well with ligroin and thrice recrystallized from aqueous alcohol. The snow-white needles then melted at 82–83° and had the following rotation in absolute alcohol.

$$[\alpha]_D^{24.5} = \frac{-1.33° \times 100}{1 \times 14.96} = -8.9°.$$

The analysis (Dumas) gave too high a nitrogen content. The material was therefore recrystallized repeatedly from both chloro-

[17] Hewitt and Kenyon found 142° at 19 mm.

[18] The highest molecular rotation found by Hewitt and Kenyon is $[M]_D^{20} = 31.0°$.

form and ligroin, and aqueous alcohol. It then melted at 82.5–83° and analyzed as follows:

7.920 mg. substance: 0.334 cc. N_2 at 763.8 mm. and 24°.
$C_{22}H_{23}O_2N$. Calculated. N 4.2.
Found. " 4.9.

The phenylurethane of this same carbinol was dextrorotatory in absolute alcohol.

$$[\alpha]_D^{23.5} = \frac{+0.83° \times 100}{2 \times 5.28} = +7.9°.$$

It was unfortunately very soluble in all organic solvents, even ligroin, and was not obtained pure. It melted at 40–41°. The nitrogen content by Dumas was too high by 1 per cent.

Levo-Ethyl-β-Cyclohexethyl Carbinol.

Reduction of Levo-Ethylphenethyl Carbinol.—10 gm. of the pure carbinol ($\alpha_D^{25} = -12.95°$) were dissolved in 90 cc. of pure glacial acetic acid and shaken with 0.2 gm. of Adams' PtO_2 catalyst with hydrogen under 3 atmospheres pressure. The hydrogen absorption was rapid and regular. After several hours, 0.3 gm. more catalyst was added. The absorption was now very rapid and regular but stopped suddenly. However, an analysis of the crude carbinol isolated, after thorough drying, showed that complete reduction had taken place. No hydrocarbon was found. The carbinol boiled at 103–108° at 0.2 mm. and had the following composition.

5.205 mg. substance: 14.835 mg. CO_2 and 6.095 mg. H_2O.
$C_{11}H_{22}O$. Calculated. C 77.7, H 12.9.
Found. " 77.7, " 13.1.

The carbinol had the following rotations.

Without solvent, 1 dm. tube, $\alpha_D^{24} = -3.89°$, $[\alpha]_D^{24} = -4.32°$, $D_4^{24} = 0.898$, $[M]_4^{24} = -7.34°$.

$$\text{In absolute alcohol } [\alpha]_D^{24} = \frac{-1.07° \times 100}{1 \times 21.20} = -5.0°.$$

$$\text{In absolute ether } [\alpha]_D^{24} = \frac{-0.82° \times 100}{1 \times 14.86} = -5.5°.$$

α-Naphthylurethane of Levo-Ethyl-β-Cyclohexethyl Carbinol.—2 gm. of the pure carbinol were treated with 2.0 gm. of β-naphthyl isocyanate and the resulting mixture warmed for 2 hours on the steam bath. In 5 minutes it had already begun to crystallize. After cooling, the mass was washed with ligroin and recrystallized twice from 95 per cent alcohol. The pure white needles melted at 114–115° and analyzed as follows:

100.0 mg. substance required 2.91 cc. 0.1 N HCl (Kjeldahl).
$C_{22}H_{29}O_2N$. Calculated. N 4.1. Found. N 4.1.

The urethane had the following rotation.

$$\text{In absolute alcohol } [\alpha]_D^{22} = \frac{+0.06^\circ \times 100}{1 \times 2.84} = +2.1^\circ.$$

In chloroform the urethane was levorotatory.

$$[\alpha]_D^{22} = \frac{-0.61^\circ \times 100}{1 \times 6.24} = -9.8^\circ.$$

Ethylstyryl Ether.

Reduction of the Levo-Ether of Levo-Ethylstyryl Carbinol.—The ether was not reduced by colloidal palladium. Thus when 2 gm. of the ether ($[\alpha]_D^{25} = -71.0^\circ$) were shaken in the usual way with palladium, only a little hydrogen was absorbed after 7 hours, and the activity fell only to -66.8° at 26°.

It was however slowly reduced by Adams' PtO_2 in 95 per cent alcohol. When 1 gm. of the ether was thus reduced, the hydrogen absorption, though slow, was steady, and about the correct volume of hydrogen was absorbed. The ether, recovered in the usual way, and dried at 100° at 1.5 mm. analyzed as follows:

3.060 mg. substance: 9.490 mg. CO_2 and 2.720 mg. H_2O.
$C_{22}H_{30}O$. Calculated. C 85.2, H 9.7.
Found. " 84.6, " 9.9.

It had the following activity in absolute ether.

$$[\alpha]_D^{25} = \frac{-0.39^\circ \times 100}{1 \times 8.06} = -4.8^\circ.$$

This activity may well be too high or even dextro for the saturated ether was not entirely free from the unsaturated ether.

Attempts to make a solid tetrabromide of the unsaturated ether failed. The theoretical amount of bromine was absorbed without evolution of hydrogen bromide. The product was an oil, soluble in ligroin. It decomposed on standing.

Attempts to distil the unsaturated ether also resulted in decomposition.

Levo-Ethylcyclohexyl Carbinol.

Reduction of Levo-Ethylphenyl Carbinol.—6 gm. of the carbinol ($\alpha_D^{23.5} = -25.68°$; $[\alpha]_D^{23.5} = -25.93°$[19]) dissolved in 50 cc. of glacial acetic acid were shaken with Adams' PtO_2 catalyst with hydrogen at 3 atmospheres pressure, until no more gas was absorbed. The platinum was then filtered off, the acid neutralized with sodium hydroxide, and the mixture extracted thoroughly with ether. After drying over anhydrous potassium carbonate the ether was pumped off and the remaining liquid distilled at 10 mm. About two-thirds came over at 39–40°. The remainder boiled at 80–85° at 10 mm. and had a strong camphor-like odor, very similar to methylcyclohexyl carbinol. In ether it had the following rotation.

$$[\alpha]_D^{21.5} = \frac{-1.39° \times 100}{1 \times 21.82} = -6.4°.$$

The carbinol was then redistilled and the fraction boiling at 85.5–86° at 9 mm. was collected. It had the following composition.

2.555 mg. substance: 7.180 mg. CO_2 and 3.405 mg. H_2O.

$C_9H_{18}O$. Calculated.	C 76.1,	H 12.7.
Found.	" 76.6,	" 13.2.

In a 50 mm. tube without solvent, it gave at 24° a levorotation of 2.93°; $\alpha_D^{24} = -5.86°$.

The liquid which constituted two-thirds of the reduction product, boiling at 39–40° at 10 mm., distilled at atmospheric pressure at 152–154°.[20] This fact and the following analysis identified the substance as propylcyclohexane.

[19] Pickard, R. H., and Kenyon, J., *J. Chem. Soc.*, **99**, 45 (1911) give as a maximum $[\alpha]_D^{20} = 27.73°$.

[20] Sabatier and Senderens found 153–154° (Sabatier, P., and Senderens, J. B., *Compt. rend. Acad.*, **132**, 568 (1901)).

4.395 mg. substance: 13.775 mg. CO_2 and 5.595 mg. H_2O.
C_9H_{18}. Calculated. C 85.7, H 14.3.
Found. " 85.5, " 14.2.

Ethylbenzyl Carbinol.

Resolution of Ethylbenzyl Carbinol.—Ethylbenzyl carbinol was easily resolved with strychnine. The salt, prepared in 95 per cent alcohol seemed at first to be more soluble in 80 to 85 per cent alcohol than in 95 per cent; but after several recrystallizations from 95 per cent alcohol this property apparently disappeared. After four such recrystallizations, the resulting phthalate (after hydrolysis) showed no increase in activity and had the following rotation in absolute alcohol.

$$[\alpha]_D^{25} = \frac{-1.17° \times 100}{1 \times 6.35} = -18.4°.$$

The carbinol obtained in the usual way boiled at 104–106° at 9 mm. and analyzed as follows:

3.365 mg. substance: 9.870 mg. CO_2 and 2.890 mg. H_2O.
$C_{10}H_{14}O$. Calculated. C 80.0, H 9.3.
Found. " 80.0, " 9.6.

In absolute ether it had the following activity.

$$[\alpha]_D^{27} = \frac{-2.90° \times 100}{1 \times 13.37} = -21.7°.$$

Without solvent, 1 dm. tube, $\alpha_D^{27} = -33.49°$, $D_4^{26} = 0.972$, $[M]_D^{27} = -51.7°$.

Levo-Ethylhexahydrobenzyl Carbinol.

Reduction of Levo-Ethylbenzyl Carbinol.—5.5 gm. of the carbinol ($\alpha_D^{27} = -33.49°$) dissolved in 50 cc. of pure glacial acetic acid were shaken with hydrogen at 30 pounds pressure with 0.5 gm. of Adams' PtO_2 catalyst. The reduction was regular and complete in about 2 hours. The reduced carbinol isolated in the usual way was thoroughly dried at 0.8 mm. at 70° for about 1 hour. An analysis of this material was as follows:

3.425 mg. substance: 9.725 mg. CO_2 and 4.025 mg. H_2O.
$C_{10}H_{20}O$. Calculated. C 76.9, H 12.8.
Found. " 77.4, " 13.2.
Calculated for hydrocarbon $C_{10}H_{20}$. C 85.7, H 14.3.

These values indicate roughly that only 8 per cent of the carbinol was reduced to the corresponding hydrocarbon. The carbinol was then distilled. The first fraction up to 102° at 10 mm. consisted of both hydrocarbon and reduced carbinol. The second and main fraction, boiling point 102–103.5° at 10 mm., was pure ethylhexahydrobenzyl carbinol which analyzed as follows:

3.660 mg. substance: 10.300 mg. CO_2 and 4.290 mg. H_2O.

$C_{10}H_{20}O$. Calculated. C 76.9, H 12.8.
Found. " 76.7, " 13.1.

In absolute ether the carbinol had the following activity.

$$[\alpha]_D^{23} = \frac{-5.08^\circ \times 100}{1 \times 20.06} = -25.3^\circ.$$

Without solvent, 1 dm. tube, $\alpha_D^{24.5} = -24.30^\circ$, $D_4^{24} = 0.900$, $[M]_D^{24.5} = -42.1^\circ$.

Dextro-Isopropylcyclohexyl Carbinol.

Reduction of Levo-Isopropylphenyl Carbinol.—5.5 gm. of the carbinol ($\alpha_D^{23.5} = -9.01^\circ$) boiling at 105–108° at 14 mm. dissolved in an alcoholic solution of glacial acetic acid (1:1) were shaken with hydrogen at 30 pounds pressure with 0.5 gm. of Adams' PtO_2 catalyst until no more hydrogen was absorbed. The carbinol was recovered in the usual way and distilled *in vacuo*. At 9 mm. there was a considerable amount of a liquid boiling at 47–48° probably the saturated hydrocarbon. The pure reduced carbinol boiled at 91–94° at 9 mm. and amounted to 2.3 gm. or about a 40 per cent yield. It had a characteristic camphor-like odor and gave the following analysis.

3.520 mg. substance: 9.945 mg. CO_2 and 3.945 mg. H_2O.

$C_{10}H_{20}O$. Calculated. C 76.9, H 12.8.
Found. " 77.0, " 12.6.

In the homogeneous state, 1 dm. tube, the carbinol was dextrorotatory, $\alpha_D^{23.5} = +2.75^\circ$.

THE FLUCTUATIONS OF THE CAPILLARY BLOOD SUGAR IN NORMAL YOUNG WOMEN DURING A TWENTY-FOUR HOUR PERIOD.

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In a recent publication Trimble and Maddock (1) have reported some experiments which indicate that young men, observed in situations unaccompanied by exercise or a recent food intake, maintain concentrations of sugar in the capillary blood which fluctuate but little from hour to hour. In view of the much greater variations which some earlier observers (2) have reported under circumstances somewhat similar it seemed that further evidence bearing upon the situation would be desirable. Also, in view of the fact that the behavior of young men only was studied in the investigation cited, it was decided to employ female subjects in our experiments. It was felt that this would be of additional interest because of some hints in the literature that greater fluctuations in the regulation of blood sugar may occur in this sex.

Methods.

The subjects for these experiments were all young women who had been in training as nurses for at least 1 year. During the period of this experiment they carried on their usual activities, merely pausing at their post of duty for us to take the blood samples. At night they slept in a ward near our laboratory. Since our laboratory is in the hospital building proper, the subjects were readily accessible and it was possible to obtain the samples exactly at the hour. The technique for obtaining the blood was essentially that employed in the investigation mentioned above. The blood samples for the first 12 hours were all collected by one of us and in the remaining period the other carried on the collec-

tion in the same manner. Thus a uniformity in obtaining and measuring the blood taken at each hour from the different individuals was insured. The sugar concentrations were measured by the Folin ferricyanide method (3). Glucose from the United States Bureau of Standards was employed in the preparation of all standard solutions.

During this experiment, the young women all partook of the same food and institutional control insured uniformity in the portions served. An approximate calculation of the carbohydrate content indicated an average intake of 45 gm. at breakfast, 40 gm. at lunch, and 67 gm. at dinner, which was decidedly lower than the values reported in the experiments upon the young men.

TABLE I.

Morning Fasting Blood Sugar of Thirty Normal Young Women.

All values are expressed in mg. per cent. The observations are recorded in horizontal lines in the order in which they were obtained.

97	91	97	96	91	89
101	89	87	94	90	94
91	90	100	94	88	94
86	86	94	89	88	100
96	98	89	92	92	95

Mean of this series = 92.6 mg. per cent.
" deviation = ± 4.1 mg. per cent.
" age = 21.2 ± 2.4 years.
" weight = 58.2 ± 9.1 kilos.

Results.

Table I presents the average concentration of capillary blood sugar of thirty normal young women who had been fasting for a number of hours. It will be noted that the mean of the series (92.6 ± 4.1 mg. per cent) is very close to that (93.6 ± 4.6 mg. per cent) found in the investigation in which the young men were subjects.

Table II presents the hourly fluctuations of the capillary blood sugar of young women living on their normal diet and engaged in their usual activities, and needs little comment. Attention is directed particularly to the great regularity of concentrations shown by the young women while in bed and asleep. Each seems

TABLE II.

Hourly Capillary Blood Sugar Concentrations of Normal Young Women.

Mean age 21.6 ± 2.8 years; mean weight 58.0 ± 11.0 kilos.

Subject......	G. E-t.	B. T-n.	M. Y-g.	D. G-r.	C. F-k.	B. B-y.	R. F-h.	Average.
Time.	Sugar concentration, mg. per cent.							
a.m.								
7.00	91	97	93			112	105	100
7.10–7.30, breakfast.								
8.00	102	104	96	109	150	123	119	115
9.00	93	90	93	109	91	98	101	96
10.00	95	95	98	114	106	106	99	102
11.00	96	96	88	138*†	98	100	106	97
p.m.								
12.00		90	91		103	102	101	97
12.10–12.40, luncheon.								
1.00	96	91	85	150	161	109	123	116
2.00	114	118	91	108	150	108	111	114
3.00	105	86	92	94	90	105	98	96
4.00	95	100	94	98	103	101	97	98
5.00	93	98	88	98	147*‡	103	98	96
5.10–5.40, dinner.								
6.00	148	108	130	156	162	103	93	129
7.00	132	116	119	116	95	114	91	112
8.00	102	108	99	102	93	114	102	103
9.00	95	110	90	104	134‡	93	99	103
10.00	97	94	89	98	132‡	105	98	102
11.00	88§	91§	83§	99§	102	108	115	98
a.m.								
12.00	87	95	85	94	102	107	112	96
1.00	85	89	88	90	104	99§	98§	93
2.00	91	91	81		103§	94	91	92
3.00	89	94	83	93	104			93
4.00	88	96	90	108*‖	103	98	95	95
5.00	88	92	92	105*	105	96	95	95
6.00	90	97	87	110*	106	97	94	95
Mean values for periods of complete rest in bed.								
Mean.....	88	94	86	94	104	97	95	
" deviation.	1.9	2.9	3.8	3.7	1.3	1.9	2.5	

* Excluded in computing average.

† Ate an orange.

‡ Ate a piece of candy.

§ In bed and asleep.

‖ On duty for emergency operation.

TABLE II—*Concluded.*
Menstrual History.¶

Subject.....	G. E-t.	B. T-n.	M. Y-g.	D. G-r.	C. F-k.	B. B-y.	R. F-h.
	+20 −13	+3 −28	+30 −1	+8 −25	+5 −19	+7 −22	+30 0

¶ + Days elapsed after onset of preceding menstrual period; − days before onset of menstrual period following this experiment.

to maintain her own characteristic level on concentrations from which the variations are not great. The mean values found for individual subjects range from 88 to 104 with the majority at 94 to 95. The mean of forty-three observations upon seven young women in a state of complete rest gives 94.0 ± 4.6 mg. per cent which agrees closely with the mean value (94.48 ± 3.5 mg. per cent) reported after 62 observations upon nine young men (1). In view of the great preponderance of sugar concentrations falling between 90 and 100 mg. per cent found for resting and fasting men and women it would seem reasonable to consider this as the basal level (at least for young people) when the Folin ferricyanide procedure is employed upon whole blood.[1] The tendency of the average to return to this region as the effect of food intake passes off (noted in both investigations) emphasized this also. Thus as data accumulate it seems more and more probable that the basal blood sugar level of normal people falls within relatively closer limits than has been the general conception. Values which previously have been considered as just within the upper and lower limits of a rather wide range clinically established may really represent situations in which the subjects were outside of basal conditions. If this interpretation of a rather narrow range of basal sugar concentrations can be maintained then it will become possible to make more accurate analyses of the physiological factors which influence transportations of carbohydrates.

The case of D. G-r. presents one feature of especial interest. She was called after 3.00 a.m. for an emergency operation which required considerable hurrying around and activity on her part

[1] Pierce and Scott (4) have recently subjected several long series of observations upon fasting blood sugar concentrations as measured by the Shaffer-Hartmann and by the Folin-Wu procedures to statistical analyses and have stressed the predominating importance of this same region.

for several hours. The sharp jump from 94 (resting average) to 108 (activity average) suggests that moderate activity produces a slight elevation of the blood sugar. This is in line with some of the observations made upon the young men (1).

Data with reference to the menstrual periods of the young women are included in Table II though Okey and Robb (5) who have studied this factor extensively have reported "there seems to be no ground for conclusion that there is a consistent cyclic variation in the fasting blood sugar level in women." It will be noted that our subjects though few in number are not concentrated in any one portion of the cycle.

SUMMARY.

The concentrations of sugar (total reducing substances) in the capillary blood of normal young women has been determined by the Folin micro method both for fasting individuals and for a group engaged in their normal activities over a period of 24 hours. Each individual subject maintained a very constant concentration level while at rest in bed. The average level found for the entire group was 94 mg. per cent, and the majority of the individuals had values close to this. The data collected for young women are in close agreement with those previously reported for young men. Therefore it seems justifiable to conclude that when the Folin micro procedure is used for measuring the sugar content of the whole blood and the subject is in a basal condition the normal fasting values should lie within or very close to the range 90 to 100. It is emphasized that the basal range for blood sugar values probably extends over a smaller area than has been the generally accepted conception.

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THE NEUTRAL FAT OF BEEF LIVER AND OTHER TISSUES.

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In a study of the lipids of beef tissues already reported (1) it was found that the fatty acid content of the "neutral" fat fraction (acetone-soluble) averaged considerably below that required for true fat. Thus, the fatty acid percentages found for liver fat were 70, for kidney fat 85, for pancreas 86, and for lung 75, while the percentage yield of fatty acid for true fat would be about 95. Theis (2) obtained the same fatty acid percentage values for the fat fraction of beef liver. It was obvious that this fraction in both cases contained a good deal of something that was not fat and that a more discriminating examination was called for. Especially in the case of the liver was an accurate knowledge of the nature of the fat desirable because of the important place assigned to that organ in our present conceptions of fat metabolism.

The basis for the general belief that the liver has a special function in the metabolism of fat, may be outlined as follows: Large accumulations of fat have been observed in the liver under a variety of conditions, such as poisoning with various substances—phosphorus, phlorhizin, chloroform, bacterial toxins, or alcohol—and as a result of fasting, hemorrhage, pregnancy, or a high fat diet. The material on this subject has been reviewed by Rosenfeld who has repeated much of the experimental work (3). His conclusions are that the fat present in the fatty liver is fat from the body stores and that the cause of the mobilization is a low glycogen content of the liver. He found that the mobilization might be prevented or reversed by feeding glycogen-forming substances and did not take place if the fat stores were empty.

Leathes became interested in these facts and began a series of investigations with various collaborators resulting in the generali-

zations which constitute the present belief regarding the early stages of the metabolism of the fatty acids. Having noted that the fatty acids of tissues, and especially of the liver, were considerably more unsaturated than the fatty acids of the food or of the fat stores, he concluded that the function of the liver in fat metabolism was to desaturate the fatty acids of the food fat, in that way preparing them for their further oxidation in the organism. With Meyer-Wedell (4) he found that after feeding cod liver oil (I.N. 150) the livers of rats and cats contained fatty acids of a much higher iodine number (up to 215) than the fatty acids of the fat fed, which fact he believed to be evidence that the liver had desaturated the fatty acids of the cod liver oil. No other organs were affected. Joannovics and Pick (5), stimulated by Leathes' work, repeated his experiments with dogs and obtained similar results. They found that the fatty acids of both phospholipid and neutral fat of the liver had a higher iodine number than the fat fed. They found, however, that an Eck fistula prevented the changes, a fact which connected the changes with the portal circulation and therefore probably with intestinal absorption. The percentage of phospholipid in the organ did not change, therefore the increased iodine values of the phospholipid fatty acids must have resulted from a replacement of some of the phospholipid fatty acids by ones of a higher degree of unsaturation taken from the fat fed. The high values disappeared after 25 hours. The interpretation made by Joannovics and Pick was somewhat different from that of Leathes. They thought that the high values were the result of *selection* of the more highly unsaturated fatty acids from material carried by the portal vein, probably absorbed from the intestine. They conceived the idea that the function of the liver was to saturate the highly unsaturated fatty acids. Recently Sinclair (6) found, as had Joannovics and Pick, that feeding cod liver oil to cats did not alter the phospholipid percentage but did result in an immediate increase in the iodine number of the phospholipid fatty acids of the liver and intestinal mucosa. In Sinclair's experiments the iodine values obtained were always below those of the oil fed. He thought that the changes were connected with the mechanism of fat absorption, the phospholipid acting as an intermediate stage between fatty acids and fat.

Other workers under the inspiration of Leathes' idea attacked various phases of the problem of liver function in fat metabolism. Mottram became interested in determining whether desaturation was a general function of liver (7) and studied the conditions in the liver and muscles of a cold blooded animal, the plaice, a flatfish. He found in this animal that the fatty acids of the liver had a lower iodine number (average 171) than those of the muscles of the fish (208) or than those of its food (average 197). His conclusion was that in these animals either the liver did not desaturate the fatty acids (rather the reverse) or if the differences were due to selection then the selection was of saturated acids. If there was desaturation it was masked by other changes, possibly synthesis of saturated fatty acids from the glycogen abundantly present in the livers of these animals. He is thus in agreement with Joannovics and Pick. Taken together the results of Mottram and of Joannovics and Pick indicate that the liver may act as a regulator of the degree of unsaturation of the fatty acids presented to it and therefore as a regulator of the degree of unsaturation of the fat carried by the blood to the tissues.

Raper (8) in the effort to provide an answer to the objection that the high values obtained for the liver fatty acids might be the result of selection by the liver of the more highly unsaturated acids from the absorbed fat rather than the result of desaturation, carried out a series of experiments with coconut oil. This fat is characterized by a very low content of unsaturated acid (I.N. 8) and by a high percentage of volatile fatty acids, characters by means of which its fate after absorption could be followed. Raper examined the volatile fatty acids of the livers of cats which had been fed coconut oil and those which had not and obtained evidence that the liver had desaturated some of the volatile acids. Perhaps from the nature of the case, his results were not very striking but he believed them sufficient to establish his point.

To determine whether the liver was the only organ in which desaturation took place or to what extent other organs were concerned, Imrie (9) made a series of examinations of human organs obtained at autopsy. He found as had Rosenfeld that the extra fat in the fatty livers could be explained as the result of an infiltration of stored fat. He found however that when the fat content did not exceed 6 or 7 per cent, the desaturation by the liver

was stimulated by the extra fat, as shown by the fact that the iodine values obtained were slightly greater than they would have been if infiltration alone had taken place. When the fat content was above 6 or 7 per cent, the iodine values were slightly below what would have been expected, indicating that the desaturating power of the liver was inhibited by the excessive load of fat. In the case of the heart and kidney, the iodine values found were always greater than would have been expected if only infiltration had taken place.

In a later paper (10) Imrie gave data to show that qualitatively the heart behaved quite similarly to the liver with regard to desaturation of mobilized fat, although the liver because of its greater capacity, was quantitatively much the more important.

Most of the highly unsaturated fatty acids of the liver are ordinarily found in the phospholipids and to determine whether the liver desaturated the fats as well as the phospholipids, Kennaway and Leathes (11) examined the "fat" fraction of the liver lipids, that is the acetone-soluble material. It was saponified and the nature of the fatty acids studied. The acetone-soluble material contains cholesterol and other "unsaponifiable" substances and also, as their analyses show, considerable material containing nitrogen and phosphorus (acetone-soluble phospholipid), so that what they analyzed as fat contained considerable amounts of non-fat. After saponification they did not separate the "unsaponifiable" matter so that the fatty acids of the fat fraction as separated contained unsaponifiable substances as well as fatty acids, with the result that the yield of fatty acid was higher than it should be, while the iodine number was affected to an unknown extent by the cholesterol and other unsaponifiable matter present. The iodine number found was, however, so much higher than that of the stored fat of the animal that their conclusion that the liver did desaturate the fats seems amply justified.

Theis (2, 12) brought evidence in support of a hypothesis that there is normally a definite relation between phospholipids and fat in the livers of animals and that in abnormal conditions this relation is departed from. Thus he found the phospholipid to fat ratio in normal beef livers was 55:45 and in normal rabbit livers 57:43. In an abnormal beef liver the ratio was found to be 29:71 with the phospholipid percentage much reduced. In

rabbits poisoned with phosphorus the ratio was found to be 4:96 with very low values for phospholipid. In insulinized rabbits ratios as low as 16:84 were found (2, 12). In our own published work on beef livers obtained by the use of the same procedure as Theis used, the average value for the ratio was found to be considerably higher (73:27), a value which fits in better with the results of MacLean and Williams (13) who found that the phospholipid of pig liver constituted 82 per cent of the liver lipids and expressed a doubt as to whether there was present any true fat at all. The differences in value for the ratio between our work and that of Theis rest mainly in the fat fraction (although our phospholipid values were about 20 per cent higher). Both investigations suffer from the same fault—that the fat fraction as analyzed contained much that was not fat. For these reasons a more critical examination of the fat fraction was decided upon and the present work is an attempt to isolate and examine the real neutral fat of beef livers and certain other beef tissues.

EXPERIMENTAL.

Livers and other tissues from freshly slaughtered animals were prepared and the lipids extracted as described previously (14). The phospholipid was precipitated from an ether solution with acetone, purified, aliquots taken for weight and iodine number determination, and the remainder used for other purposes. The acetone-ether solution containing the fat was then treated by one or other of the following procedures.

A. The acetone was distilled off, and the fatty material dissolved in the minimum amount of dry ether and reprecipitated with acetone. The insoluble material was separated, the acetone solution evaporated, and the fatty mass suspended in water from which it was precipitated by excess of acetone and a little NaCl (15). The precipitate was treated with an excess of warm acetone which dissolved most of it. The insoluble residue was added to the material obtained by reprecipitation from ether with acetone and analyzed as phospholipid. The acetone solution containing most of the fat was concentrated and two or more fractions separated and analyzed. The results from this procedure were not satisfactory, although they were better than those reported in previous work. The values as averages are reported as Series I in

Tables I and II. In twelve samples of beef liver the average percentage recovery of fatty acids was 85 with an average iodine number of 77.

B. The most suitable procedure for purifying the fat fractions was essentially that used by MacLean (15) and was as follows: After separation of the main body of the phospholipids, the acetone-soluble portion containing the neutral fat was freed from acetone and ether, and the syrupy liquid was added drop by drop with shaking to a large excess of dry acetone in centrifuge tubes. Between 600 and 700 cc. of acetone were used for each precipitation, there being about 75 cc. in each 100 cc. centrifuge tube. The first few drops of syrup added caused a haziness; on addition of a little more the solution became cloudy and finally a flocculent precipitate separated. The appearance of the precipitate indicated that about enough syrup had been added for the amount of acetone. The precipitates were centrifuged down, the acetone distilled off, and the precipitation in acetone repeated as long as any material came down. When no further precipitation occurred with acetone alone, a drop or two of a saturated alcoholic solution of calcium chloride was added and this procedure was repeated as often as a precipitate was obtained.

After no more phospholipid was precipitated by acetone in the presence of calcium chloride, the syrup was poured into boiling water and boiled for a few minutes to remove water-soluble material, after which it was allowed to stand in the refrigerator overnight. The fat solidified, sometimes forming a layer on top of the water, while at other times there was a suspension of solid particles throughout the water.

The fat was filtered off by suction, dissolved in 50 to 75 cc. of acetone, and put in the refrigerator for several hours, usually overnight. The material which separated out was separated by centrifuge and analyzed as Fraction I. The remainder, after being freed from acetone, was analyzed as Fraction II. Occasionally a third fraction was separated.

The fractions were dissolved in dry ether, made up to a volume of 50 cc., and aliquots taken for weights. The remainder was saponified for at least 8 hours with normal sodium ethylate. The unsaponifiable portion was separated from the fatty acids in the usual way, the fatty acids dissolved in petroleum ether, and the usual determinations made on aliquot portions.

TABLE I.

Neutral Fat of Liver and Other Organs.

	Sample No.	Fat, total weight.	Fatty acids.								
			Fraction I.			Fraction II.			Fraction III.		
			Weight.	Fat.	I.N.	Weight.	Fat.	I.N.	Weight.	Fat.	I.N.
		gm.	gm.	per cent		gm.	per cent		gm.	per cent	
Liver. Series II.	5	2.21	0.98	88	62	0.89	89	102			
	7	3.08	2.14	86	73	0.48	78	105			
	1	3.32	2.86	86	97						
	6	3.76	2.82	94	76	0.56	70	116			
	11	4.0	2.25	90	78	1.18	80	104			
	10	4.15	0.73	76	122	1.78	97	75			
	3	4.54	1.70	83	73	2.25	92	97			
	4	4.59	3.43	85	77	0.36	76	123			
	2	4.76	3.57	95	87	0.83	80	107			
	9	13.12	1.55		102	7.3	80	61	2.43	91	65
	8	15.10	0.72	91	95	6.3	99	62	7.11	91	74
	12	30.86	12.5	96	60	9.0	93	48	7.60	95	64
Series I (12 samples), average values.		5.75	2.92	88	74	1.98	83	98			
Heart.	4	2.03	1.42	72	59	0.16	97	83			
	9	3.89	3.24	91	41	0.38		81			
	3	4.93	3.30	89	67	0.90	87	87			
	8	5.10	4.32	91	50	0.28	83	82			
	6	5.76	4.00	90	65	0.69	85	91			
	7	5.81	5.13	95	60	0.38	88	85			
	1	6.19	4.23	91	52	1.30	86	71			
	2	6.38	5.00	95	58	0.87	83	78			
	5	7.81	7.47	94	57	0.34	90	97			
Lung.	2	0.62	Trace.			0.53	88	90			
	3	0.37	"			0.34	83	95			
	6	1.26	0.22	85	64	0.92	92	78			
	5	1.33	0.59	70	77	0.40	80	84			
	1	1.59	0.95	95	66	0.45	76	89			
	4	2.17	1.30	81	68	0.45	74	86			
Kidney.	1	2.00	1.10	84	67	0.68	92				
	2	2.56	1.68	97	54	0.72	87	88			
	3	3.16	2.40	81	68	0.60	87	101			
	4	4.36	2.20	90	62	1.50	70	72			
	5	4.68	3.24	88	69	0.74	90	98			

The acetone-soluble phospholipids were washed with acetone, dissolved in dry ether, and then centrifuged to remove the salts. The phospholipid was then dried, weighed, and analyzed as usual.

The analyses of the fat fractions of liver, heart, lung, and kidney are given in Table I.

DISCUSSION.

Liver.—The samples prepared by the second procedure are arranged in Table I in the order of their fat content (Series II). Those from Series I are reported as averages only. In the two series reported, the amount of fat was never very large, being over 1 per cent in only three of the twenty-four samples and averaging 0.59 per cent for all. Since, as shown by the fatty acid recovery, the samples still contained some impurity the true percentage of fat would be still lower.

The percentage recovery of fatty acids from the fat Fraction I average 86.3 and from Fraction II 85. In Samples 9, 8, and 12, which may be considered separately because of their high content of fat, the fatty acid recovery was 92 per cent. In Series I the average fatty acid recovery for Fraction I was 88, for Fraction II 83 per cent of the weight of fat taken.

The average iodine numbers for Series II were, for Fraction I 83, for Fraction II 91. For the last three samples of Series II, Samples 9, 8, and 12, the average iodine number was 70, indicating that the excess fat in these samples was mobilized depot fat which in this animal has an iodine number of about 60. For Series I the average iodine value for Fraction I was 74, for Fraction II 98. The iodine number for all the samples thus averaged at least 20 points higher than the depot fat of this animal.

Since the objection may be raised that the impurity still present in most of the fat fractions may be responsible for the high iodine values it should be stated that there were in all, sixteen fractions which yielded over 90 per cent of fatty acids, and had an average yield of 93 per cent. These fractions, which were therefore practically pure fat, had an average iodine number of 75. In this number were included six large fractions containing over 4 gm. of fat, which were apparently mobilized tissue fat since their iodine numbers averaged 66. Excluding these, the ten remaining samples had an average iodine number of 82.

These results, which are free from many of the objections which might be raised against the work of Kennaway and Leathes, agree with their findings that the neutral fat of liver is considerably more unsaturated than that of the fat stores, and support their conclusion that the liver desaturates the neutral fat brought to it. One reservation should however be made, and that is regarding the possibility of selection by the liver of the more unsaturated portions from the fat brought to it (see "General discussion" below).

Heart.—Fraction I contained most of the fat and in it the percentage recovery of fatty acids averaged 90; the average iodine number was 56. Fraction II was always small in amount; the fatty acid recovery averaged 88 and the iodine number 84. Most of the neutral fat of the heart was therefore of the stored or depot type and only a relatively small proportion was of the more unsaturated variety found in the liver.

Lung.—The amount of fat in lung tissue was found to be very small and, as judged by the percentage recovery of fatty acids, relatively impure. Such as it was, it was considerably more unsaturated than the depot fat.

Kidney.—In the kidney, as in the heart, most of the fat came out in Fraction I, the recovery of fatty acids was fairly good (88 per cent), and the iodine number (average 64) indicated that it again was of the depot type of fat. Fraction II was relatively small in amount but had a much higher iodine value than Fraction I.

General Discussion.—Of all the organs examined the liver was the only one which contained noteworthy amounts of neutral fat more unsaturated than the depot fat and in most livers the unsaturated fat was the characteristic fat present. Only when the amount of fat was unusually large (1 per cent or over) did it show the characteristics of the depot fat. These results are in agreement with the belief of Kennaway and Leathes and with Leathes' general conception of the function of the liver in fat metabolism, that the liver desaturates the fat transported to it from the stores and that it is the only organ which does so to any notable extent. However, before complete adherence can be given to Leathes' conception of the liver as an organ which desaturates the fatty acids, the possibility must be kept in mind that the more highly unsaturated fatty acids found there may be the

TABLE II.

Relation of Neutral Fat to Other Lipids.

The results are expressed in gm. per kilo.

	Sample No.	Neutral fat, weight.	Phospholipid.		Ratio, phospholipid to neutral fat.	Unsaponifiable, weight.	Cholesterol, weight.	Ether-insoluble.
			Weight.	I.N.				
Liver. Series II.	5	2.21	35.25	82	94:6	0.47		
	7	3.08	37.68	85	92:8	1.08		
	1	3.32	40.87	77	93:7	1.26		
	6	3.76	38.73	83	91:9	1.59		
	11	4.00	44.87	70	92:8	1.40	1.05	
	10	4.15	41.17	70	91:9	1.57	1.19	
	3	4.54	37.83	75	89:11	1.69		
	4	4.59	39.21	79	90:10	1.11		
	2	4.76	52.59	74	92:8	0.87		
	9	13.12	41.17	71	75:25	3.56		
	8	15.10	39.82	78	73:27	2.80		
	12	30.86	36.68	76	54:46	2.77	2.0	
Average			40.0	76.6				
Series I, average values.		5.75	35.75	82	86:14	0.73		
Heart.	4	2.03	19.95	88	90:10	0.72	0.48	
	9	3.89	14.15	85	82:18	0.75	0.55	
	3	4.93	22.00	92	82:18	0.87	0.60	
	8	5.10	16.22	89	74:26	0.86	0.60	
	6	5.76	20.17	98	80:20	0.85	0.58	
	7	5.81	19.20	96	77:23	0.88	0.56	
	1	6.19	15.80	93	72:28	1.52	1.02	
	2	6.38	16.14	82	71:29	1.32	0.90	
	5	6.81	19.07	92	70:30	1.01	0.75	
Average			18.28	90.5				
Lung.	2	0.62	14.75	84	96:4	2.31	0.95	3.76
	3	0.37	15.56	77	98:2	1.85		3.82
	6	1.26	13.50	84	91:9	2.16	1.73	4.53
	5	1.33	13.48	83	91:9	2.14	1.75	3.84
	1	1.59	16.27	80	91:9	1.93	1.30	3.60
	4	2.17	14.12	77	86:14	2.58		5.95
Average			14.61	81				

TABLE II—*Concluded.*

	Sample No.	Neutral fat, weight.	Phospholipid. Weight.	Phospholipid. I.N.	Ratio, phospholipid to neutral fat.	Unsaponifiable, weight.	Cholesterol, weight.	Ether-insoluble.
Kidney.	1	2.00	18.24	93	90:10	1.74	1.37	3.93
	2	2.56	19.56	96	88:12	2.78	1.60	4.00
	3	3.16	19.08	94	86:14	2.95	1.25	3.00
	4	4.36	19.74	91	82:18	2.41	2.16	3.80
	5	4.68	20.73	76	82:18	2.05	1.76	2.70
Average....................			19.42	90				

result of selection from fat passing through it from the intestine, as claimed by Joannovics and Pick, suggested by Mottram, and implied by Sinclair's work. Such a removal from the circulation of the more highly unsaturated fatty acids which are thought by some to be toxic would be in line with known detoxifying functions of the liver toward other intestinal products such as those resulting from intestinal putrefaction. It, however, implies absorption of fat directly into the portal blood stream, which so far has not been demonstrated. Attention should also be directed to the results of Mottram and of Sinclair, noted above, that the fatty acids of liver are not always more unsaturated than those of the fat fed. The small amount of unsaturated fat in the other tissues may be accounted for either as formed there or transported from the liver.

The small percentage of neutral fat found in most of the livers examined (in eighteen out of twenty-three samples examined it was below 0.7 per cent) indicates that in Leathes' words 'the liver keeps up with its task [of desaturation]" and does not act as a fat store, which latter statement is in agreement with the recent results and conclusions of Terroine (16).

The regularly high percentage values for the fat of beef liver found by Theis may possibly be explained as due to a different nutritional condition in his animals, since it is well known that fasting brings about a movement of fat to the liver.

Relations of Neutral Fat to Other Lipids in Liver and Other Organs.

The samples in Table II are arranged as in Table I in the order of their neutral fat content. Since all the livers examined were

apparently normal and since the phospholipid values were all within normal limits, there is no reason to believe that the variable values for fat indicate anything more than the variation to which the neutral fat of the liver and other organs is known to be subject,—the result of different nutritional conditions.

Neutral Fat and Phospholipid.

The phospholipid content of the livers of Series II was remarkably constant. In eleven of the twelve samples it did not depart more than 10 per cent from the average value, and the greatest departure from the average was 30 per cent in Sample 2. Similar constancies may be noted in the case of the lung and kidney while the variations in the values for the heart were considerably wider. This constancy in values for the phospholipids of various organs has been emphasized by Mayer and Schaeffer and later workers of the same school (17, 18).

The phospholipid values for all the organs in this series were higher than those found in our earlier work. Thus, the average for liver in Series II was 40 gm. per kilo, while in the earlier work it was 31. In Series I of the present report, where the separation was better than in the earlier work, the average was 35.75 gm. per kilo. (Theis' values for beef liver phospholipid averaged 25.3.) Similar higher results were obtained for heart, 18.28 gm. per kilo as compared with 16.4 in the earlier work, 14.6 as compared with 12.4 for lung, 19.4 as compared with 14.3 for kidney. The higher values obtained in the present work are undoubtedly due in part at least to a better separation.

The iodine numbers of the phospholipids of the organs in Series II show that the values for the liver phospholipids are lowest (average 76.6), heart and kidney are highest (90), and lung intermediate (81). The values for both heart and liver are notably lower than those obtained in the earlier work (present work, liver 77, heart 91; earlier work, liver 84, heart 101). In Sinclair's work on cats (19) the iodine number of the phospholipid fatty acids of liver was found to be higher than that of the fatty acids of any other organ. These differences in iodine values in the same organ at different times may probably be explained by the fact discovered by Sinclair that the fatty acids of tissue phospholipids change in response to the fatty acids of the food fat. The response in the

case of the liver is immediate (within a few hours after feeding), while in the case of the other organs, it is much later; possibly it occurs only in the course of the wear and tear and replacement process. The fact that the liver phospholipid has a *lower* iodine value than the other organs in the present series is notable and calls for comment. If the liver is the organ which is responsible for the desaturation of the fatty acids, one might expect the phospholipids in it to have a higher iodine value than those found in other tissues, as was actually found to be the case with the neutral fat and as Sinclair found in cats. But it is also obvious that if the liver desaturates the fatty acids of the phospholipids it must start with fatty acids of a lower degree of unsaturation and at any one time there may be present phospholipids in various degrees of unsaturation, the average of the values of which would be below that of the final product. Presumably this final highly unsaturated phospholipid is distributed to the tissues such as the heart and kidney and would explain the high iodine numbers found there.

Phospholipid to Fat Ratio.—As compared with the constancy of the percentage values for the phospholipid, the variability of the neutral fat values is to be noted in all the tissues but most markedly in the liver. The ratio of phospholipid to neutral fat in this series was therefore not constant but widely variable. In the liver the ratio (expressed in parts of 100) in eight of the twelve samples was 90:10 or higher, in one of the other four it was as low as 54:46. This variability of the ratio is due to variability of the neutral fat, not of the phospholipids, and may be referred to the special function of the liver in fat metabolism which is the basis of Leathes' hypothesis. The ratio in the kidney and lung is relatively constant and high; in the heart it is variable, since the heart stores a variable amount of fat.

Unsaponifiable Substance.—In all tissues examined the unsaponifiable substance was quite variable, being generally higher when the neutral fat was high but with no marked regularity.

Cholesterol.—In some samples of liver and in all the samples of the other tissues, cholesterol was determined colorimetrically in the unsaponifiable substance. As noted in Table II it generally constituted 60 per cent or over of the unsaponifiable matter. No regularity in the phospholipid-cholesterol relation could be observed.

Ether-Insoluble Substance, "White Matter."—In the lung and kidney when the total ether-soluble lipids were treated with dry ether, there was always some white material which either did not dissolve in the dry ether or which separated from it on standing. In the liver and heart it was present in traces only. This material, probably a mixture of several substances including sphingomyelin and cerebrosides, is of unknown significance. It is found in much larger amounts in extracts of brain.

Acetone-Soluble Phospholipid.—The phospholipid recovered from the acetone solution averaged for the liver 1 gm. per kilo of moist tissue, for heart 1.18 gm., for kidney 1.6 gm., and for lung 1.7 gm. The fatty acid per cent of this phospholipid was within 2 points above or below 60 per cent. The iodine number of the fatty acids was 95 to 100 for all organs but the lung, in which it was 84. The acetone-soluble phospholipid was thus not markedly different from that separated in the ordinary way.

Free Fatty Acids.—The acetone-soluble fraction always contained some free fatty acid—approximately one-third of its weight. The nature and significance of the free fatty acid in these organ extracts will be discussed in a subsequent paper.

SUMMARY.

The neutral fat of beef liver was found to be considerably more unsaturated than the fat of the fat depots or of the other organs examined. This finding is in agreement with that of Kennaway and Leathes and supports Leathes' conception of the liver as a desaturating organ for the fatty acids. However, the possibility cannot be excluded that the presence of more highly unsaturated fats in this organ may be due to selection of these substances from the blood, possibly as part of the protective function of the liver.

The percentage of fat in most of the livers examined was variable and small both absolutely and in relation to the phospholipid content, and for this reason the phospholipid to fat ratio was high (average about 86:14). Occasional high fat values in normal samples of all tissues emphasize the normal variability of the fat of the liver, and other organs.

While the fat content of the organs was variable the phospholipid content was quite constant for each organ and characteristic of the organ.

The iodine number of the liver phospholipid was found to be lower than that of the other organs examined.

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THE ACTION OF ACTIVATED ERGOSTEROL IN THE CHICKEN.

I. THE EFFECT ON THE CALCIUM AND INORGANIC PHOSPHORUS OF THE BLOOD SERUM.

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It was observed early in the study of rickets that changes in the bone structure were accompanied by changes in the amount and proportion of calcium and phosphorus of the blood serum. Park (1), in his discussion of the etiology of rickets, states that the first detectable signs are probably a diminution of the inorganic phosphorus or calcium of the blood. It has been amply demonstrated with rats and other animals that certain changes in the amount and proportion of calcium and phosphate in the blood serum indicate rickets. It is important to recognize that the proportion of calcium to phosphorus is not due solely to the amount of these elements in the diet, but also to the antiricketic factor which is needed for their proper utilization.

The effectiveness of activated ergosterol in the treatment of rickets is well known, but there seems to be a question as to its influence on the calcium and phosphorus of the blood serum when amounts above the therapeutic level are fed. One cause of the variance in reported results is that the potency of the activated ergosterol used was not always determined. The potency may vary within wide limits. It is possible that in one case a mg. of activated ergosterol may equal in potency 700,000 mg. of cod liver oil, whereas in another case the potency may be only 50,000. Therefore it is necessary that in all reports on the effect of an overdose of ergosterol, the actual potency must be taken into consideration.

Hess and Lewis (2) found that large doses of activated ergosterol given to normal infants or animals, *e.g.* 1 mg. daily for a week, led to a marked hypercalcemia. They reported as high as 16 to 18 mg. of calcium per 100 cc. of serum. The inorganic phosphate at times was raised to very high levels. Later Hess, Weinstock, and Rivkin (3) reported that large amounts of irradiated ergosterol fed to young rats on a diet almost free from calcium and high in phosphorus caused the calcium of the blood to increase 50 per cent or more.

Smith and Elvove (4), working with rabbits, found that both calcium and inorganic phosphorus increased under the influence of large doses of irradiated ergosterol.

Sobel and Claman (5) pointed out that when rickety children who showed a normal blood calcium and a low phosphorus (2.1 to 4.1 mg. per cent), were fed from 1 to 4 mg. of ergosterol daily, their phosphorus increased after a few weeks to from 5.4 to 6.9 mg. per cent. The potency of the activated ergosterol used was not stated.

A considerable literature has sprung up concerning the effect of overdosage of irradiated ergosterol. It would serve no purpose to review all the observations reported in this country and abroad. All are agreed that activated ergosterol is exceptionally effective in the cure and prevention of rickets.

For the production of experimental rickets rats have been the animals most used. In birds, unlike rats, leg weakness can be produced by the absence of the vitamin alone, regardless of the calcium and phosphorus content of the diet.

It is our purpose to observe the changes in the calcium and inorganic phosphorus of the blood serum of birds fed diets with different ratios of calcium and phosphorus but deficient in the antiricketic factor, and in addition to note the changes in the calcium and phosphorus when a high dosage of activated ergosterol is added to the diets.

EXPERIMENTAL.

The birds were single comb White Leghorn cockerels, selected at an age of 18 weeks. From the time of hatching the birds were on a complete diet to which was added 2 per cent of cod liver oil. They were allowed free range in sunlight until the beginning of the experiment. They weighed approximately 1.4 kilos each.

Twenty birds were put in five lots of four birds each. Four of the lots received the following basal ration. The components were ground fine and intimately mixed.

79 parts yellow corn-meal.
10 " wheat gluten.
5 " casein, crude.
5 " linseed oil meal.
1 part NaCl.

The following mineral supplements were made to this ration: Lot I received the basal ration plus no mineral supplement. Lot II received 6 per cent c.p. $CaCO_3$ added to the basal ration at the expense of the corn. Lot III received 1 per cent $CaCO_3$ and 5 per cent $NaH_2PO_4 \cdot 2H_2O$ added at the expense of the corn. Lot IV received 6 per cent c.p. $Ca_3(PO_4)_2$ added at the expense of the corn. Lot V received McCollum's rickets-producing Diet 3143 (6).

Table I gives the calcium and phosphorus content of the different diets.

TABLE I.

Calcium and Phosphorus in Experimental Diets.

Diet No.	Ca	P	Approximate ratio Ca:P.
	per cent	*per cent*	
I	0.108	0.341	0.32
II	2.502	0.321	8
III	0.502	1.311	0.38
IV	2.424	1.521	1.6
V	1.271	0.253	5

The experimental pens were so arranged that no direct sunlight was available. Fresh tap water was given to drink. The birds were bled from the heart by means of a hypodermic needle into a glass syringe. Determinations of calcium and phosphorus were made on each bird separately. The blood was transferred to a 15 cc. centrifuge tube, allowed to clot, and the clot was broken by means of a small glass rod. It was then centrifuged at a high speed for 5 minutes. The serum was withdrawn by a pipette and a determination made for calcium by the Clark-Collip (7) method, and for inorganic phosphates by the method of Benedict and Theis (8).

The birds were kept on the respective diets for a period of 8 weeks. Weekly determinations of calcium and phosphorus of the serum of each individual bird were made.

At the end of the 8th week, 4 per cent of an activated ergosterol solution, potency 1000 times that of average cod liver oil, was incorporated in the diet. The birds were bled 2, 5, 10, 15, and 35 days after the addition of the activated ergosterol. Determinations were made for calcium and phosphate on each bird.

Table II gives the average amount of calcium and phosphorus of the birds of each group for the 8 weeks on diets deficient in vitamin D and again after the period of treatment.

TABLE II.

Calcium and Inorganic Phosphorus of Blood Serum of Young Cockerels.

The results are expressed as mg. per 100 cc. of serum.

Time.	Lot I. No mineral supplement. Ca:P, 0.32.		Lot II. 6 per cent $CaCO_3$ added to diet. Ca:P, 8.		Lot III. 5 per cent $NaH_2PO_4 \cdot 2H_2O$ and 1 per cent $CaCO_3$ added to diet. Ca:P, 0.38.		Lot IV. 6 per cent $Ca_3(PO_4)_2$ added to diet. Ca:P, 1.6.		Lot V. McCollum's ricketic Diet 3143. Ca:P, 5.	
	Ca	P	Ca	P	Ca	P	Ca	P	Ca	P
Start.	10.4	5.1	10.4	5.5	10.3	5.2	10.7	5.3	10.6	5.2
1 wk.	10.5	5.4	16.6	5.4	10.9	4.7	11.1	5.6	12.8	4.5
2 wks.	10.8	5.3	15.2	3.5	9.7	4.7	10.4	5.2	12.2	3.7
3 "	10.0	4.0	14.4	2.4	9.9	4.4	9.7	4.3	11.1	3.7
4 "	10.2	4.4	14.1	2.6	9.4	4.9	10.2	4.5	11.7	3.8
5 "	9.9	4.4	13.6	2.2	8.7	5.4	10.1	4.1	11.4	3.8
6 "	9.0	4.0	12.1	2.2	8.0	5.9	9.7	3.9	10.8	3.6
7 "	8.8	3.5	11.9	2.0	7.5	6.2	9.8	4.3	10.0	3.8
8 "	8.5	3.8	11.5	2.0	7.3	7.1	10.1	4.8	9.4	3.6
Solution of irradiated ergosterol added (= 4000 per cent cod liver oil).										
2 days	9.6	4.1	13.3	2.2	8.3	5.7	9.5	4.9	10.9	4.6
5 "	9.3	4.6	15.1	2.5	9.3	4.8	10.1	4.8	10.8	5.1
10 "	10.1	3.9	13.8	3.0	9.9	5.0	11.7	5.2	10.5	4.7
15 "	10.7	4.4	15.7	3.0	11.6	4.8	11.6	5.4	10.6	5.4
35 "	10.5	4.4	15.0	2.5	11.8	6.1	13.0	4.8	11.7	4.9

The activated ergosterol was standardized by the line test method (9).

DISCUSSION.

The method used for obtaining the blood did not seem to cause any ill effects in the birds. Within a few hours it was impossible to tell that the birds had been bled. Out of the twenty birds

started on the experiment, only one was killed by this method of bleeding. It is admitted, however, that there is a possibility that the continual bleeding may have had some effect on the calcium and phosphorus of the blood serum, but we feel that since in most cases there were at least 5 days between bleedings, the effect on these elements would be small. Nevertheless, since all the birds were bled under the same conditions, the results are comparable. Birds of this age take longer to show evidence of calcium and phosphorus deficiency than younger birds, since older birds have greater opportunity to store vitamin D. Since our birds had complete diets, with cod liver oil and sunlight, before the start of the experiment, there can be no doubt that the calcium and phosphorus were normal at the beginning. It will be noted that in most cases the calcium at the start of the experiment was 10 to 11 mg. per 100 cc., and the phosphorus was 5 to 6 mg. There were only small variations in the calcium and phosphorus in birds at the age used when they had received a complete diet.

Since the experimental diets varied in their calcium and phosphorus content, it is to be expected that the blood serum would vary in these elements, especially when there was a lack of vitamin D in the diet.

In the birds on the high calcium, low phosphorus diets, Diets II and V, the serum calcium was increased for the 1st week and then there was a steady decline. The serum calcium of the birds receiving 6 per cent $CaCO_3$ in the rickets diet was, after 8 weeks, still appreciably above normal, but it was lower than it was after the 1st week. Thus it appears that when a diet relatively high in calcium and low in phosphorus, and lacking in vitamin D, is fed to birds the serum calcium tends to remain above normal for a considerable period. On the other hand, the inorganic phosphorus of the birds on such diets declined steadily, the more marked decline taking place in the birds receiving the more calcium. When activated ergosterol solution equivalent to 4000 per cent of cod liver oil (assayed on rats) was added to these diets, there was a marked increase of serum calcium of both groups within 2 days. The serum calcium of the birds receiving 6 per cent calcium carbonate reached the maximum in approximately 15 days. The serum calcium of the birds receiving 3 per cent calcium carbonate (McCollum Diet 3143) was still increasing after 35 days. The serum

phosphate of the birds on these two diets, after activated ergosterol was added, is of particular interest. The phosphorus of the diets was very low and varied approximately 0.07 per cent. The serum phosphate of the birds of Lot II, in which the ratio of calcium to phosphorus in the diet was approximately 8:1, was exceptionally low. When the activated ergosterol was added to the diet, there was only a slight increase in the inorganic phosphorus of the blood and this element did not reach normal within the 35 days. On the other hand, the inorganic phosphate of the birds in Lot V (Diet 3143), in which the ratio of calcium to phosphorus was 5:1, became approximately normal 15 days after the addition of ergosterol. On the 35th day, however, the phosphorus was lower than on the 15th day. One would conclude from these results that when the ratio of calcium to phosphorus in the diet is exceedingly high, activated ergosterol has little effect on the inorganic phosphorus of the blood serum.

Both the calcium and phosphorus in the diet of the birds of Lot I were low, 0.108 and 0.341 per cent respectively. It is interesting to note that both the calcium and phosphorus decreased in the serum of the birds in the absence of vitamin D. On the addition of activated ergosterol the amount of calcium in the serum approached normal. There was also an increase of phosphate in the serum. Whether this condition would persist for any length of time is doubtful because the requirements of calcium and phosphorus are greater than the amounts present in this diet. We have data to show that when young chicks are fed a diet similar to that of Lot I, with the addition of 2 per cent of cod liver oil, they develop leg weakness during the 8th to 10th week.

The birds in Lots III and IV received diets containing calcium and phosphorus in ratios of approximately 0.38:1 and 1.6:1 respectively. The diet of Lot III was higher in calcium than Lot I and higher in phosphorus than either Lots I, II, or V. The diet of Lot IV contained approximately the same percentage of calcium as Lot II but more phosphorus. It is interesting to note that there was a steady decline in the serum calcium of the birds of Lot III on the rickets diet, but a steady increase in the phosphorus. On the addition of activated ergosterol the tendency was for both calcium and phosphorus to approach normal.

On the other hand, in the serum of the birds in Lot IV, when the diet was ricketic, there was only a slight variation in both the calcium and phosphorus. The addition of activated ergosterol to the diet increased the calcium but did not affect the phosphate to any appreciable extent. There was a slight decrease in calcium on the 2nd day after the addition of the ergosterol, but afterwards a steady increase.

It will be noted that when activated ergosterol was added to any of the diets, there was a change in the calcium and phosphate of the serum within 2 days. The maximum change usually occurred by the 15th day.

Thus it is evident that the proportion and amount of calcium and phosphorus in the diet must be considered when blood studies are made on chickens. When a diet is fed containing an exceedingly high proportion of Ca:P, it may be impossible for activated ergosterol so to regulate the calcium and phosphorus metabolism as to give normal proportion and amounts of these elements in the blood.

SUMMARY.

1. In the serum of birds receiving a diet lacking in vitamin D and having a ratio of calcium to phosphorus of 8:1, the calcium increased during the first week and remained above normal for 8 weeks; the inorganic phosphorus decreased rapidly during the first 3 weeks and then remained approximately stationary. The addition of activated ergosterol (= 4000 per cent cod liver oil) caused a marked increase of the calcium but only a slight increase of the phosphorus. When the ratio of calcium to phosphorus was 5:1, the calcium remained above normal for 5 weeks but there was a steady decrease of the phosphorus, though not as much as where the ratio was 8:1. The addition of the activated ergosterol caused both calcium and phosphorus to become approximately normal.

2. When activated ergosterol (= 4000 per cent cod liver oil) was fed to birds on high calcium rickets-producing diets, there were marked changes in the calcium of the blood serum within 48 hours.

3. The serum calcium and phosphorus of birds of the age used in this experiment (18 weeks) remained approximately normal

when they received a rickets-producing diet in which the ratio of calcium to phosphorus was 1.6:1. The addition of activated ergosterol had no marked effect on either the calcium or phosphate of the serum until after 15 to 35 days, when an increase in serum calcium was noted.

4. When the birds received a diet low in both calcium and phosphorus, both the serum calcium and inorganic phosphorus decreased. On the addition of activated ergosterol there was a marked increase in serum calcium but only a slight increase in phosphate. After 35 days the calcium of the serum was approximately normal, yet the inorganic phosphorus remained subnormal.

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THE ACTION OF ACTIVATED ERGOSTEROL IN THE CHICKEN.

II. THE PREVENTION OF LEG WEAKNESS.

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The effectiveness of activated ergosterol in the treatment of rickets in children and rats is well known, and a considerable literature has accumulated with reference to its use. Little is known concerning the action of activated ergosterol on leg weakness in chickens, although cod liver oil and sunlight are recognized as effective prophylactic agents.

Kreitmair and Moll (1) in a study of the sensitiveness of several species to overdoses of activated ergosterol found that the chicken could withstand larger doses than any other of the animals used. They stated that the proper dose for curing rickets in the rat is 0.0001 mg. daily, whereas 0.01 mg. is required for the chicken. The vitamin D potency of the activated ergosterol was not reported. Since the potency may vary widely, their work can be accepted only on a comparative basis.

In a previous article (2) we demonstrated that large doses of activated ergosterol (= 4000 per cent of cod liver oil) greatly influenced the calcium and inorganic phosphorus of the blood serum of birds on ricketic diets. The effect was governed by the amount and proportion of calcium and phosphorus in the diet. These results were to be expected if there is a relationship between the leg weakness of chickens and the rickets of rats. Practically all investigators state that 2 per cent of cod liver oil is sufficient to prevent leg weakness. Thus the amount of activated ergosterol fed in our experiment was 2000 times the cod liver oil vitamin D requirement.

The object of this experiment was to ascertain the vitamin D

requirement of the growing chick when activated ergosterol was the source of this factor.

EXPERIMENTAL.

The birds were single comb White Leghorn chicks, selected at the time of hatching. They came from the eggs of breeders which had received a complete diet, with 2 per cent of cod liver oil and access to sunshine. 50 chicks were divided into five lots of ten each, and placed on the following basal ration, to which various vitamin D additions were made:

56	parts	yellow corn.
10	"	half skim milk powder.
10	"	wheat bran.
10	"	linseed oil meal.
10	"	wheat gluten.
3	"	$CaCO_3$
1	part	NaCl.

Lot I received the basal ration without supplement. Lot II received the basal ration plus 2 per cent of cod liver oil. Lot III received the basal ration plus (A) 0.02 per cent of activated ergosterol solution 100 D (= 2 per cent of cod liver oil by assay on rats), (B) the same amount of activated ergosterol diluted to 2 per cent with corn oil. Lot IV received the basal ration plus (A) 0.2 per cent of activated ergosterol solution 100 D (= 20 per cent of cod liver oil), (B) the same amount of activated ergosterol diluted to 2 per cent with corn oil. Lot V received the basal ration plus 2.0 per cent of activated ergosterol solution 100 D (= 200 per cent of cod liver oil).

The rations were made up weekly. This eliminated as far as possible the loss of potency through oxidation. The vitamin D potency of the activated ergosterol was determined by the line test method with rats (3). The birds were housed in such a manner that they had no access to direct sunlight. Fresh tap water was given to drink.

The birds were kept on their respective diets for 10 weeks. They were then killed, and a femur removed from each. The bones were cleaned thoroughly, crushed, extracted first with alcohol for 12 hours, and finally with ether for 16 hours. They were dried, weighed, and ashed in a muffle at 700–750°. The

percentage of ash was calculated on the basis of the dried extracted bone.

Table I gives the percentage of ash of the bones of the birds on the different diets.

TABLE I.

Ash of Chicken Bones after Different Vitamin D Supplements.

Lot I. Basal ration without vitamin D supplement.		Lot II. Basal ration plus 2 per cent cod liver oil.		Lot III. Basal ration plus 0.02 per cent activated ergosterol solution 100 D (= 2 per cent cod liver oil).		Lot IV. Basal ration plus 0.2 per cent activated ergosterol solution 100 D (= 20 per cent cod liver oil).		Lot V. Basal ration plus 2 per cent activated ergosterol solution 100 D (= 200 per cent cod liver oil).	
Bird No.	Bone ash.	Bird No.	Bone ash.	Bird No.	Bone ash.	Bird No.	Bone ash.	Bird No.	Bone ash.
	per cent		*per cent*		*per cent*		*per cent*		*per cent*
1	35.00	1	46.58	A1	40.92	A1	45.51	1	48.16
2	38.46	2	49.20	A2	37.00	A2	44.03	2	48.00
3	40.84	3	47.98	A3	44.77	A3	44.40	3	46.01
4	36.57	4	48.42	A4	43.02	A4	47.76	4	45.66
5	44.00	5	47.00	B5	40.00	A5	44.04	5	46.63
6	40.49	6	47.48	B6	40.24	B6	44.03	6	46.00
7	41.78	7	46.00	B7	42.43	B7	42.20	7	48.12
8	39.94	8	45.90	B8	40.00	B8	44.78	8	48.21
9	42.00	9	46.42	B9	42.50	B9	43.20	9	48.26
10	39.90					B10	44.00		
Average.	39.89		47.22		41.21		44.39		47.23

DISCUSSION.

Leg weakness in birds is a dietary disease and is evidenced by a low calcium or phosphorus content of the bones. Since tricalcium phosphate is the chief constituent of bone, a determination of ash is an excellent criterion of the condition of the bone. It is well known that a low ash is indicative of leg weakness.

The ash of the bones of the birds in Lot I, those which did not receive any vitamin D supplement, was lower than the ash of those receiving 2 per cent of cod liver oil, but in all probability these birds did not suffer from severe leg weakness. It is possible that if the experiment had been continued longer, a lower ash of the bones of the birds of this group would have been obtained. These results may indicate that the Ca:P ratio of this diet was optimum or nearly optimum for the growing chick.

If a basal diet had been used in which the Ca:P ratio was different from that of this experiment, a lower ash of the bones might have resulted in all the lots. It is likely that a different Ca:P ratio in the diet would influence the action of activated ergosterol in the mineral metabolism of growing birds.

It appears from Table I that when the vitamin D equivalent of 2 per cent of cod liver oil was given to growing birds in the form of activated ergosterol, the ash of the bones was appreciably lower than the ash of the birds receiving cod liver oil. Nevertheless, the bones of the birds which received the vitamin D as activated ergosterol showed more ash than those receiving no vitamin D at all. It was necessary to administer a quantity of activated ergosterol equivalent, by assay on rats, to 200 per cent of cod liver oil, to produce the effect of 2 per cent of cod liver oil.

SUMMARY.

Activated ergosterol, in concentrated or dilute solutions, was much less effective than the rat equivalent amount of cod liver oil in preventing leg weakness in chickens.

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MERCURY DERIVATIVES OF CYSTEINE.

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INTRODUCTION.

In a paper dealing with the optical activity of cysteine Andrews (1) reported that the method of preparation of cysteine described by Harris (2) failed to give a completely reduced product. This method consisted in precipitating cystine with mercuric sulfate in sulfuric acid solution with subsequent treatment of the precipitate with hydrogen sulfide. The specific rotation of the product indicated approximately 85 per cent reduction. It seemed plausible that the function of the hydrogen sulfide was not only to precipitate the mercury but to reduce the newly liberated cystine to cysteine according to the reaction:

$$2H^+ + S^= + RSSR^{++} \rightarrow 2RSH^+ + S^0$$

Thus sulfur would be precipitated and the cysteine formed by a fairly simple reaction and, although hydrogen sulfide is without action when directly applied to cystine, the simultaneous formation of mercuric sulfide and liberation of the cystine might be regarded as catalyzing the reduction of the latter. However, the above explanation was weakened by the fact that repeated attempts to indentify free sulfur in the precipitate from the action of hydrogen sulfide always failed.

It appeared desirable, therefore, to investigate the composition and properties of the precipitate of cystine with mercuric sulfate and the present paper records the results of such an investigation. Since these results were obtained, however, a paper by Vickery and Leavenworth (3) on the preparation of cysteine through the formation of a silver compound has recently appeared. In it these authors describe a reaction by which the original precipitate

formed by silver sulfate is not a compound of cystine but of cysteine, the latter having probably been formed by a mutual oxidation-reduction reaction which produces a small proportion of cysteic acid as well. Thus, that portion of the cystine which has been converted to silver cysteine is capable of direct regeneration to the free amino acid by either hydrogen chloride or hydrogen sulfide. The mechanism which Vickery and Leavenworth advance for the formation of the silver compound is supported by several observations which the present authors have made on the mercury compound. These observations are discussed in detail below.

EXPERIMENTAL.

The samples of the cystine-mercuric sulfate precipitate were prepared by dissolving 10 gm. of *l*-cystine in 2500 cc. of 5 per cent H_2SO_4 and adding an excess of the $HgSO_4$ reagent (10 per cent $HgSO_4$ in 5 per cent H_2SO_4). The flocculent white precipitate produced by an excess of the reagent was always preceded by the formation of a gray compound when smaller proportions of mercuric sulfate were used. This gray compound, while apparently stable in the cold, quickly gave place to the white flocculent precipitate when more of the reagent was added.

Two preparations made with excess mercuric sulfate were used for the analyses described below. Both preparations were carefully washed, but washing was difficult due to the cohesive, amorphous properties of the precipitate. Therefore, the precipitate was transferred from the filter to a large beaker and there carefully washed with distilled water. The suspension was then filtered in successive portions, each portion being again washed when on the filter. When the entire suspension had been so treated, the precipitate was again transferred to the beaker and the process was repeated. This method of washing was continued until the test for sulfate was almost negative, large quantities of water being used. The washings no longer gave a positive test for Hg^{++} after 2.5 liters of distilled water had been used, and after the first 15 or 20 liters the amount of $SO_4^{=}$ present in the filtrate showed a marked decrease, but thereafter it appeared roughly constant and the washings were always faintly acid. The samples were dried in a vacuum desiccator over P_2O_5.

The hygroscopicity of the compound (see below) made drying a slow process, while the use of higher temperatures for drying was precluded by the instability of the compound. At temperatures of 250–300° the white compound blackens instantly, with formation of metallic mercury. This decomposition is initiated at a temperature of 120° and even below.

Methods of Analysis.—Total nitrogen was determined by Kjeldahl analysis, 10 per cent sodium sulfide solution being used to precipitate the mercury after digestion was complete. Blanks were run on all reagents used.

Mercury was determined as follows: A 0.5 gm. sample was subjected to Kjeldahl digestion; the solution was cooled and diluted to 200 cc. It was then heated to boiling and hydrogen sulfide passed in for about 1 hour, with the solution gently boiling. It was then allowed to stand 48 hours, filtered through a Gooch filter, and washed free from sulfates. Very little free sulfur was present and this, after drying, was extracted with carbon disulfide. The precipitate was dried to constant weight at 110°.

Sulfur was determined by precipitation as barium sulfate, oxidation being accomplished with fuming nitric acid. The results of a number of analyses of both samples averaged as follows:

Sample I. N 2.87, Hg 61.86, S 7.55.
" II. " 3.04, " 60.34, " 7.22.

The individual figures used in making these averages varied by ± 0.03 per cent for N, ± 0.05 per cent for Hg, and ± 0.10 per cent for S.

Difficulties in drying have already been noted. The hygroscopic properties of the dried compound were also evidenced by a rapid gain in weight during the weighing of samples. To determine the hygroscopicity of the compound a sample was placed on a weighed watch-glass in the balance case and weighed at intervals. The gain in weight was at first fairly rapid; 5 hours exposure to open air caused an increase in weight of over 0.5 per cent. On further exposure the sample gained in weight more slowly but quite steadily, particularly on damp days.

Optical Activity.—To determine the completeness of precipitation of the cystine-mercuric sulfate precipitate the original filtrate was tested for optical activity. A rotation of +0.15° was ob-

served, while the initial washings gave a rotation of +0.13°. Similar results were obtained on other such filtrates; for example, +0.11°, +0.05°, +0.10°, and +0.07°. It will be noted that in view of the possible formation of cysteic acid such rotations would be expected.

Potentiometric Titration.—For comparison with the nitrogen to mercury ratio obtained analytically (see below) several potentiometric titrations were made. 0.5 gm. of *l*-cystine were dissolved in 50 cc. of 5 per cent H_2SO_4 and the 10 per cent $HgSO_4$ reagent was added from a burette. The voltage was measured by a type K potentiometer, with a standard Weston cell and calomel half-cell. Both bright and platinized platinum electrodes were used, but the

TABLE I.

Potentials Resulting from Titration of 0.5 Gm. of Cystine with 10.12 Per Cent $HgSO_4$ Solution. Platinum Black Electrode; 25°.

10.12 per cent $HgSO_4$ solution.	Voltage.	10.12 per cent $HgSO_4$ solution.	Voltage.
cc.		*cc.*	
1.26	+0.566	10.28	+0.560
2.58	0.553	13.46	0.592
4.82	0.530	17.10	0.607
6.05	0.507	20.22	0.618
6.95	0.485	24.36	0.625
7.36	0.495	30.30	0.632
9.00	0.538	31.82	0.633

most satisfactory results were obtained with the latter. An agar-KCl bridge between the calomel cell and the cystine solution was necessitated by the fact that the presence of halides greatly increased the solubility of the mercury compound and gave very erratic curves. Further experiments with bromide and iodide gave parallel results; the compound is easily soluble in alkali halides leaving only, in the case of the iodide, a slight precipitate of mercuric iodide.

In the absence of any halide, a smooth and reproducible curve was obtained with a definite minimum at about 7 cc. of 10 per cent $HgSO_4$ reagent, with no indication of any other maximum or minimum. This would indicate slightly more than a 1:1 ratio of cystine to mercuric sulfate (0.5 gm. of cystine reacting with 0.62

gm. of $HgSO_4$), with no indication of a compound with the proportions shown by the analysis (see "Discussion"). The first addition of the reagent caused the formation of some precipitate, which appeared to come down at all times without delay. Table I shows the data corresponding to a typical curve of this sort. In all cases the minimum of the curve was very close to the 7 cc. point.

Gray Compound.—Mention has been made above of a gray compound obtained with smaller proportions of mercuric sulfate. Samples of this precipitate showed, under the microscope, a mass of long needle-like crystals somewhat enlarged at one end, in sharp contrast to the amorphous character of the white precipitate to which the gray changed on addition of more mercuric sulfate. These needle-like crystals were very fragile and were easily broken into irregular fragments. No further information concerning this intermediate compound has been obtained. It may, of course, represent the first step in the reaction which we postulate below: a simple mercuric dicysteine.

DISCUSSION.

The compositions recorded for Samples I and II, when calculated in terms of atomic ratios of nitrogen to mercury, yield the following:

Sample I.

$$\text{N.}\quad \frac{2.87}{14.01} = 0.205$$

$$\text{Hg.}\quad \frac{61.86}{200.6} = 0.309$$

Sample II.

$$\text{N.}\quad \frac{3.04}{14.01} = 0.217$$

$$\text{Hg.}\quad \frac{60.34}{200.6} = 0.301$$

Thus in Sample I a very definite ratio of 2 atoms of nitrogen to 3 of mercury is indicated, while in Sample II the proportion of mercury is about 8 per cent below a 2:3 ratio. If we accept this proportion, there is indicated a compound of 3 mols of mercury combined with either 1 cystine or 2 cysteine molecules. In such a compound, the mercury may replace the hydrogen of carboxyl or (in cysteine) sulfhydryl groups or a mercury-containing group may be attached to one or more amino groups. If we assume a mercury compound analogous to the silver cysteine described by Vickery and Leavenworth we may expect to have 1 mercury atom substituted in the two sulfhydryl groups of 2 cysteine molecules

with two more mercury-containing groups attached as indicated above. These latter groups may be either $HgSO_4$ or a hydrolysis product (HgO or $Hg(OH)_2$). Consideration of the various possible compounds of this type shows that the percentage composition recorded above is probably best explained by the assumption that we are dealing with a mixture of two or more of the following compounds.

A. $HgC_6H_{12}O_4N_2S_2\,(HgSO_4)\,(Hg(OH)_2)$.
Mol. wt. 972.15, N 2.88, Hg 61.90, S 9.89.

B. $HgC_6H_{12}O_4N_2S_2\,(HgSO_4)\,(Hg(OH)_2)\cdot H_2O$.
Mol. wt. 990.17, N 2.83, Hg 60.78, S 9.71.

C. $HgC_6H_{12}O_4N_2S_2(2Hg(OH)_2)(2H_2O)$.
Mol. wt. 946.14, N 2.96, Hg 63.60, S 6.78.

The low percentage of sulfur found as compared with the calculated values for Compounds A and B suggests that a larger proportion of sulfate was probably hydrolyzed out. The persistence of sulfuric acid in the wash water supports this view. However, the above structures are advanced for the sole purpose of meeting the needs of the analytical data at hand.

In this connection it is interesting to note that a similar mercury-cysteine compound was prepared by Brenzinger (4) directly from cysteine and mercuric chloride. The analysis of this compound also showed a proportion of 2 nitrogen to 3 mercury atoms and indicated a compound analogous in all respects to our compound with mercuric sulfate. Brenzinger commented on the ease with which hydrochloric acid is lost by hydrolysis and assigned a constitution in which 1 mercury atom replaced the 2 hydrogen atoms of the sulfhydryl groups, while the other 2 were combined with the amino groups.

It is obvious that a number of rearrangements of each of these compounds are possible. In opposition to the analytical findings, the curve of electrode potential *versus* cc. of mercuric sulfate (Table I) shows such a pronounced break at a 1:1 ratio of cystine to mercury (or 2:1 cysteine to mercury) as to leave no doubt about definite compound formation at this ratio. If we assume a compound of cystine with mercuric sulfate, there appears to be no reason why the introduction of the 1st atom of mercury should produce any sharper change than the 2nd or the 3rd. But if we assume a compound of *cysteine* with mercury, it is clear that the introduction of the 1st atom of mercury has ac-

companied the reduction of cystine to cysteine whereas the others have probably combined in a purely additive way. Thus we should expect a sharp break in the curve only from the first step: the reduction of cystine.

The dextrorotatory solutions which, as noted above, were obtained when the precipitate from the action of mercuric sulfate on *l*-cystine is filtered appear to substantiate the assumption of cysteic acid as a by-product of the reaction. The specific rotation of cysteic acid as reported by Friedmann (5) is about +8.25.

In some studies on the separation of cystine and tyrosine Plimmer (6) reported that, as determined by Kjeldahl, mercuric sulfate removed 81 per cent of the cystine and left 19 per cent unprecipitated. This figure agrees satisfactorily with that calculated from the equation postulated by Vickery and Leavenworth, 83.3 per cent.

In addition, the mechanism outlined provides a satisfactory explanation for the absence of free sulfur in the mercuric sulfide precipitate when the mercury compound is decomposed by hydrogen sulfide.

SUMMARY AND CONCLUSION.

The composition of the compound formed by the action of mercuric sulfate on cystine has been determined and a general structure has been assigned to it. This structure, corresponding to that assigned by Vickery and Leavenworth to the compound formed by silver sulfate on cystine, is probably that of a mercury dicysteine containing 2 more atoms of mercury. This hypothesis is supported by other experimental observations, such as the absence of free sulfur from the precipitate obtained when the mercury compound is treated with hydrogen sulfide, the dextrorotatory solutions resulting from the precipitation of *l*-cystine with mercuric sulfate, and the form of the electrode potential curve given by the reaction.

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THE OPTICAL ACTIVITY OF d-ARGININE.

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In a recent paper by Hunter (1) on the specific rotation of *d*-arginine the $[\alpha]_D$ value, calculated to the free base, was reported as $+26.5°$ when in acid solution with 8 mols of hydrochloric acid per mol of arginine. In the course of some work on arginine in this laboratory it has frequently been more convenient to measure its optical activity under other conditions, particularly as the pure monohydrochloride. We wish, therefore, to report the following data in the variations in $[\alpha]_D$ which this amino acid shows under a variety of other conditions.

Of the three samples of arginine hydrochloride used, two were prepared from gelatin by the method of Cox (2) and the other by the method of Vickery and Leavenworth (3). All three gave the theoretical values for both total nitrogen by Kjeldahl analysis for α-NH_2 nitrogen by the Van Slyke procedure with 4 minutes reaction at 20–22°. It was also found possible to get a fairly quantitative determination of the second NH_2 group in the Van Slyke apparatus in 1 hour at 25° provided the sample introduced consisted of not more than 4.0 mg. of arginine. These three samples also gave identical results in the polariscope and, under the conditions employed by Hunter, checked his value to within 0.1 $[\alpha]_D$ unit. The same was true of a fourth preparation which was recovered from residues of the first three. Evidently no detectable racemization results from the recovery and recrystallization of arginine hydrochloride.

In determining rotatory power, the amounts required were weighed out as accurately as possible and all dilutions were made in United States Bureau of Standards flasks. Polariscopic measurements were made in 4 dm. tubes (for discussion of use of shorter tubes see below) which had been checked with pure solutions

of sucrose and found to give the usually accepted values. A Schmidt and Haensch polariscope, sensitive to ±0.01°, was used. All readings were made with sodium D light.

The data reported were obtained chiefly from measurements of the specific rotation of arginine in solutions with varying ratios of HCl or NaOH. In most cases a 1 per cent solution (free base) was first made up and then diluted in two ways for further optical

TABLE I.

Specific Rotation of Arginine.

Concentration.	$\frac{\text{Arginine}}{\text{HCl}}$ = 1:8 mols. $[\alpha]_D^{25}$		$\frac{\text{Arginine}}{\text{HCl}}$ = 1:5 mols. $[\alpha]_D^{25}$		$\frac{\text{Arginine}}{\text{HCl}}$ = 1:1 mols. $[\alpha]_D^{25}$	
	H_2O dilution.	Acid dilution.	H_2O dilution.	Acid dilution.	H_2O dilution.	Acid dilution.
gm. per 100 cc.						
10.00	+26.5					
1.00	+26.5	+26.5	+23.0	+23.0	+17.5	+17.5
0.50	+26.5	+27.0	+23.5	+22.0	+17.5	+18.0
0.25	+26.0	+26.0	+23.0	+22.0	+16.5	+21.0
0.125	+24	+28	+24	+24	+20	+22

Concentration.	Isoelectric arginine. $[\alpha]_D^{25}$	$\frac{\text{Arginine}}{\text{NaOH}}$ = 1:1 mols. $[\alpha]_D^{25}$		$\frac{\text{Arginine}}{\text{NaOH}}$ = 1:10 mols. $[\alpha]_D^{25}$		$\frac{\text{Arginine}}{\text{NaOH}}$ = 1:30 mols. $[\alpha]_D^{25}$
	H_2O dilution.	H_2O dilution.	Alkali dilution.	H_2O dilution.	Alkali dilution.	H_2O dilution.
gm. per 100 cc.						
1.00	+12.5	+13.7	+13.7	+13.2	+13.2	+11.7
0.50	+12.0	+14.5	+14.0	+14.0	+14.5	+14.5
0.25	+12.0	+14.0	+14.5	+13.0	+14.0	+16.0
0.125	+10	+14	+14	+14	+16	+16

readings, (1) with water and (2) with acid or alkali of the same concentration as that present in the 1 per cent solution. In order to duplicate more closely Hunter's conditions a 10 per cent solution (free base) was also used, with results closely duplicating his.

Table I records the data correlating $[\alpha]_D^{25}$ with acidity (or alkalinity) and concentration of arginine. All concentrations and $[\alpha]_D$ values are expressed in terms of the free base.

The data in Table I show the variation in $[\alpha]_D$ value for different levels of arginine concentration on both sides of its isoelectric point. To obtain a more consistent measure of this variation at a lower level of arginine concentration a series of dilutions was made of a stock arginine hydrochloride solution such that each solution contained 0.200 gm. per 100 cc. (as free base) and variable ratios of HCl or NaOH. These data are shown in Table II.

TABLE II.

Specific Rotation of Arginine Solutions of Constant Concentration (0.200 Gm. per 100 Cc.).

Mols acid or base per mol arginine.	$[\alpha]_D^{25}$	pH	γ	pG
316 HCl.	+26.2			
15 "	+25.0			
9 "	+25.0			
7 "	+23.8			
5 "	+22.5			
3 "	+21.2			
2 "	+18.7	2.20		
1 "	+16.2	5.40	0.667	5.10
0.75 "	+15.6	8.60	0.707	8.21
0.50 "	+13.1	9.05	0.872	8.22
0.25 "	+11.9	9.61	0.906	8.33
Isoelectric.	+11.2			
0.30 NaOH.	+12.0	(12.50)	0.210	13.08
0.50 "	+12.5	(12.85)	0.342	13.13
1 "	+13.8			
3 "	+15.0			

The figures in parentheses are values taken from Hunter and Borsook.

In the case of a few solutions near the isoelectric point, pH determinations[1] are also included.

The calculation of the ionization constants of arginine from the optical activity data appears to be of some interest since the application of this method by Levene and coworkers (4) to alanine and similar compounds.

The method consists essentially in the determination of the degree of dissociation of the amino acid from its rotations under

[1] These were run by Mr. E. P. Laug of this department.

various pH conditions and the substitution of this dissociation value in the usual Hasselbalch equation

$$\gamma_a = \frac{M-Ma}{Mo-Ma} \qquad \gamma_b = \frac{M-Mo}{Mb-Mo}$$

$$pG = pH - \log \frac{\gamma}{1-\gamma}$$

where M = the observed molecular rotation at the pH in question, Mo = the isoelectric molecular rotation, and Ma and Mb, the molecular rotation of the completely repressed salt of the ampholyte with acid or alkali. The symbols in the Hasselbalch equation have their usual significance. In applying this dissociation constant method to arginine no account was taken of the second basic dissociation constant.

The last column in Table II shows the pG values obtained. These should be compared with the corresponding pG values calculated from the constants of Hunter and Borsook (5), which were determined by direct titration and which are the best values for this amino acid at present available. Hunter and Borsook's data give values of 8.95 and 12.85 respectively. The parenthetical pH values for two of the basic solutions are not experimental values but were merely read from the titration curve of arginine plotted according to the Hunter and Borsook constants. These figures are included only as an example of the application of optical data to this mode of calculation.

The pG values, calculated from the optical data, furnish at best only a rough approximation to Hunter and Borsook's values. It should be noted that for a substance of as low a concentration as that employed in the data in Table II, the normal error involved in reading the polariscope (±0.01°) corresponds to a difference of about 0.25 pG unit when data fairly close to the isoelectric point of arginine are involved. However, it is in this region that the figures of Hunter and Borsook are most closely approximated.

The determination of optical activity of such weakly rotatory substances is also subject to a further error if perfectly clear solutions are not used. With almost imperceptible amounts of turbidity the character of the spot of light in the polariscope may be so changed as to give a somewhat different zero point and

thus produce an erroneous reading. In such cases the amount of the turbidity interposed becomes of importance and different specific activity values may be obtained with tubes of different lengths. Readings taken in 1 dm. tubes have shown $[\alpha]_D$ values as much as 50 per cent or more above those taken on the same solution in 4 dm. tubes.

SUMMARY AND CONCLUSION.

The optical activity of *d*-arginine has been measured under a variety of conditions. The observations of Hunter (with which we are in agreement) have been extended to other concentrations and acidities.

The method of Levene and coworkers for the calculation of titration constants from optical activity data has been applied to arginine. Results in rough agreement with the direct titration data of Hunter and Borsook have been obtained.

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OXIDATION-REDUCTION POTENTIALS OF CERTAIN SULFHYDRYL COMPOUNDS.*

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In recent years a number of investigators have attempted to extend the fundamental ideas concerning oxidation-reduction potentials to systems of organic nature. In particular there have been rather elaborate studies of a number of physiologically important materials which have led to significant results, in spite of the fact that they cannot be formulated according to the laws of thermodynamics. These studies have demonstrated that oxidation and reduction in tissues is generally an irreversible process incapable of reaching the equilibrium necessary for mathematical formulation.

The work to be described in this article was suggested by the paper of Dixon and Quastel[1] who observed the potential of cysteine at an inert electrode since it was known to be a substance which exhibits a strong reducing power. They derived, on the basis of an assumed equation

$$2\ \mathrm{RSH} - 2\ e \rightleftarrows \mathrm{RSSR} + 2\ \mathrm{H}$$

the expression for the "normal reduction potential"

$$\pi = \pi_0 + \frac{RT}{F} \log (\mathrm{H}^+) - \frac{RT}{F} \log \frac{(\mathrm{RSH})}{\sqrt{(\mathrm{RSSR})}} \qquad (1)$$

---

* This article is an abstract of the thesis of E. M. Drissen submitted in partial fulfilment of the requirements for the degree of Bachelor of Science from the University of Wisconsin, in 1927. An extension of the work will be reported in another place.

[1] Dixon, M., and Quastel, J. H., *J. Chem. Soc.*, **123**, 2943 (1923).

where π = observed potential, π_0 = "normal reduction potential," parentheses represent molecular concentrations, RSH indicates cysteine or other reduced sulfhydryl compound, and RSSR indicates cystine or other oxidized sulfhydryl compound. Their experimental result, apparently amply verified by other observers, was of a somewhat surprizing nature since it was found that the reduction potentials of cysteine (and this is also true of thioglycollic acid) were quite independent of the presence or absence of the oxidized form. In other words the measurements led to the following formula.

$$\pi = \pi_0 + \frac{RT}{F} \log (\mathrm{H}^+) - \frac{RT}{F} \log (\mathrm{RSH}) \tag{2}$$

Dixon and Quastel also postulate the existence of an intermediate compound in the oxidation of cysteine to cystine.

Conant,[2] in an article dealing with the electrochemical formulation of the irreversible reduction and oxidation of organic compounds, points out in regard to the work of Dixon and Quastel, "it is uncertain whether or not the cystine-cysteine and the glutathione system are reversible and can be formulated in electrochemical terms. Dixon and Quastel, by using carefully prepared electrodes, obtained reproducible potentials with solutions of cystine and cysteine, and of glutathione and the corresponding disulfide, but the potentials were independent of the relative concentration of the two components of the system. Since dependence on relative concentrations is the most important criterion of reversibility one is inclined to believe that their potentials are not characteristic of the behavior of these systems. They suggest that we are here dealing with a new type of electrode system but the problem remains as to how one should interpret the potential of such a system. In this connection it should be mentioned that carefully prepared electrodes will often record fairly reproducible potentialsin pure buffer solutions in the absence of any oxidation-reduction systems; the E.M.F. varies regularly with the hydrogen ion concentration. It is possible that some such "accidental" potentials were the source of the electromotive force measured in Dixon's work. A further study of disulfide

[2] Conant, J. B., *Chem. Rev.*, **3**, 1 (1926).

systems with a series of reversible reagents of known potential seems advisable."

The possibility mentioned that the potentials of Dixon and Quastel could be accidental seems now to be definitely disproved. Important information on this point is given by Michaelis and Flexner.[3]

It appeared to us that another explanation was possible and even more probable, and our experimental work was planned accordingly. If the cysteine could be oxidized to some soluble sulfhydryl compound other than cystine the potential at an inert electrode will be a function of the concentration *both* of the cysteine and of this oxidized form other than cystine, in addition to the hydrogen ion concentration of the solution.[4] In this work the attempt was made to treat the system by the ordinary titration method with the idea of determining the apparent oxidation potentials. It was decided to run a series of electrometric titrations oxidizing cysteine with selected oxidizing agents, at definite pH values, recording the variation of potential, and to determine if possible the value of E_0' from the curves in which the observed potentials are plotted against cc. of oxidizing agent added. In every case there was obtained a typical "S" shaped curve, similar to the familiar type which results when ferrous ammonium sulfate is oxidized by potassium chromate. Such a curve corresponds to the form of Equation 1. The values of E_0 may be determined if an assumption concerning the number of electrons involved in the chemical change can be made. E_0' is the observed potential at the point of 90.5 per cent oxidation of cysteine and E_0 is the "apparent oxidation potential."

Normal oxidation potential being defined as "the measure of the

[3] Michaelis, L., and Flexner, L. B., *J. Biol. Chem.*, **79**, 689 (1928).

[4] This is not equivalent to the suggestion made by Dixon and Quastel which is quoted here. "If the [RSSR] term is to be regarded as constant it is obvious that this cannot represent the concentration of cystine. It seems almost necessary, therefore, to postulate the possible presence of an intermediate compound which would be the "true" oxidized form of cysteine and is transformed irreversibly into cystine. In such a case the concentration of this intermediate oxidized substance would appear to be maintained at a constant value—as though the solution in presence of cysteine must be saturated with respect to this particular compound."

tendency of an ion to pass from a higher to a lower state of oxidation,"[5] the equation for the supposed reaction, for the purpose of deriving the formula, may be written

$$RSSR + 2\ H^+ + 2\ (e) \rightleftarrows 2\ RSH \tag{3}$$

The expression for the observed potential then becomes

$$E_h = \frac{RT}{2\,F}\log K - \frac{RT}{2\,F}\log\frac{(RSH)^2}{(RSSR)\ (H^+)^2} \tag{4}$$

where E_h = observed potential, or

$$E_h = E_0 + \frac{RT}{2\,F}\log\frac{(RSSR)\ (H^+)^2}{(RSH)^2} \tag{5}$$

E_0 being the normal oxidation potential. On simplification the latter equation becomes

$$E_h = E_0 + \frac{RT}{F}\log\frac{\sqrt{(RSSR)}\ (H^+)}{(RSH)} \tag{6}$$

Separating the last term, we have

$$E_h = E_0 + \frac{RT}{F}\log\ (H^+) + \frac{RT}{F}\log\frac{\sqrt{(RSSR)}}{(RSH)} \tag{7}$$

If then, the observed voltage, E_h, is taken at the point of 90.5 per cent oxidation, *i.e.* 90.5 per cent oxidized form and 9.5 per cent cysteine, the last term becomes equal to zero and drops out. The observed voltage at this point is designated as E_0' and Equation 7 becomes

$$E_0' = E_0 - \frac{RT}{F}\ \text{pH} \tag{8}$$

or, at 25°, the temperature of the titrations,

$$E_0' = E_0 - 0.0592\ \text{pH} \tag{9}$$

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[5] Creighton, H. M. J., and Fink, C. G., Principles of electrochemistry, New York, 2nd edition (1928).

Apparatus.

The electrode or titration vessel used was a slightly modified form of the one used by Clark[6] and is diagrammed in Fig. 1. The cell is a gold-calomel cell of about 200 cc. capacity. The calomel half-cell is normal KCl, its single electrode potential being assumed to be +0.283 volt. The mechanical stirrer was operated by an air motor.

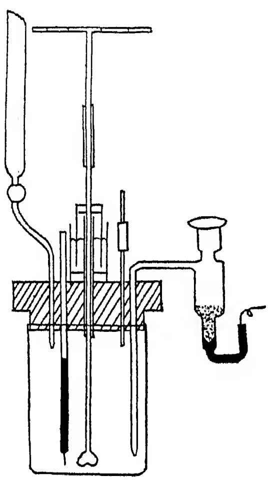

FIG. 1.

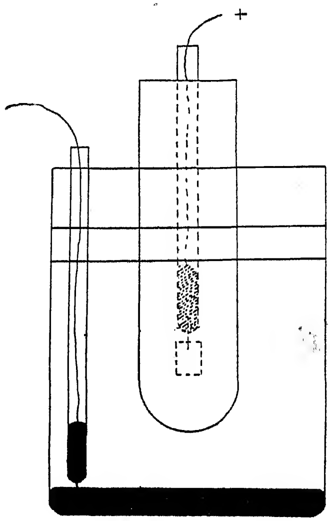

FIG. 2.

FIG. 1. Titration vessel.
FIG. 2. Type of cell used in the preparation of the cysteine.

Potentials were measured by means of a Leeds and Northrup student type of potentiometer with wall galvanometer.

Method.

The cysteine was prepared by a slight modification of the electrolytic method of Andrews.[7] The cell used is diagrammed in

[6] Clark, W. M., *Pub. Health Rep., U. S. P. H. S.*, **37**, 4 (Reprint 834) (1923).

[7] Andrews, J. C., *J. Biol. Chem.*, **69**, 209 (1926).

Fig. 2. The catholyte consisted of distilled water with the cystine, about 0.8 gm., suspended in it, no particular attempt being made to put the latter in solution. The anolyte, contained in a porous cup, was approximately 0.1 N HCl. The voltage employed was 90 volts and the time of reduction was about 48 to 50 hours. Air was excluded by the simple expedient of covering the solution with mineral oil. The concentrations of cysteine produced were

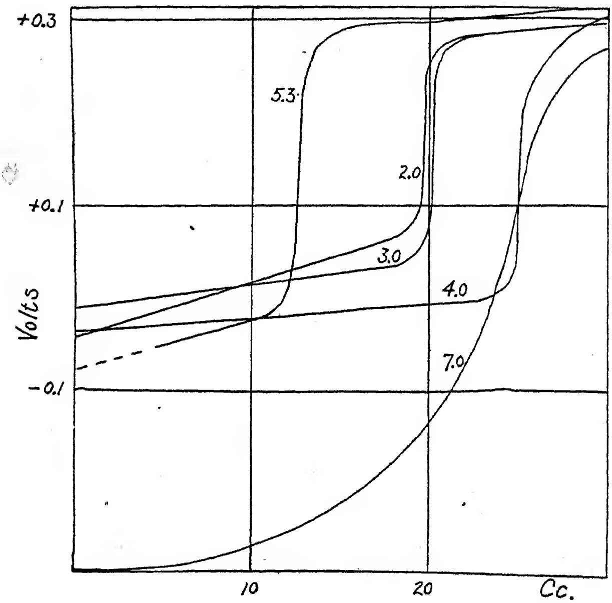

FIG. 3. Titration of cysteine with 0.04 N iodine. The figures on the curves represent the pH.

approximately 0.02 N. It was not necessary to prepare solutions of accurate concentration, inasmuch as the type of curve and the E.M.F. at a relative stage of oxidation are independent of concentration.

There were placed in the electrode vessel 60 cc. of buffer solution and 40 cc. of the solution of cysteine from the reduction cell. The reagents used for titration were 0.04 N solutions of iodine, potassium iodate, and potassium dichromate. Five titrations with iodine, at pH values 2.0, 3.0, 4.0, 5.3, and 7.0 were made.

With potassium dichromate, four titrations were made with each reagent, at pH values of 2.0, 3.0, 4.0, and 5.3, and with potassium iodate titrations were made at pH values of 2.0, 3.0, and 5.3. Typical experiments are shown graphically in Fig. 3.

In addition thioglycollic acid solutions were oxidized with iodine at pH values of 3.0, 5.2, 6.1, and 7.3, with potassium permanganate at a pH value of 3, and with molecular oxygen at a pH value of 6.1.[8]

The initial voltage of the cell was recorded and the titer added from the burette, 1 cc. at a time, readings being taken after each addition. As the end-point approached, the readings were taken more frequently. About 1 minute was necessary for the attainment of equilibrium with the potassium iodate and potassium dichromate; iodine showed more rapid attainment of equilibrium as it also showed sharper end-points.

Results.

The values of E_0' were read from the graphs, being the observed potentials at points 90.5 per cent of the distance between the initial point of the curve and the end-point. They were corrected to the hydrogen electrode by adding 0.283 volt to each reading. From these values the value of E_0, the apparent normal oxidation potential, was calculated in each case from the formula

$$E_0 = E_0' + 0.0592 \text{ pH} \qquad (10)$$

Example.—The end-point in the titration with KIO_3, at pH 2.0, was determined by graphing $\frac{dP}{dcc.}$, where dP is the difference of potential and $dcc.$ is the unit difference in titer added. When this end-point was determined at 23.4 cc. the point of E_0' was 90.5 per cent of 23.4 cc. or 21.2 cc., and E_0' was read as +0.089. Corrected to the hydrogen electrode this becomes +0.372, or E_0' (corrected).

Substituting in Equation 10,

$$E_0 = +0.372 + 0.0592\ (2.0) = 0.490$$

[8] The titrations of the thioglycollic acid solutions were made by Mr. E. K. Fischer of this laboratory. The authors take this opportunity to thank him for his kindness in allowing us to use his data.

The equation holds where the assumed reaction involves two electrons, and the values of E_0 thus calculated for the values of E_0' obtained with KIO_3 and $K_2Cr_2O_7$ are given in Table I.

TABLE I.

Cysteine.

pH	End-point.	Point of 90.5 per cent oxidation.	E_0'	E_0' (corrected).	E_0
			Potassium iodate.*		
	cc.				
2.0	23.4	21.2	+0.089	+0.372	+0.490
3.0	23.4	21.2	0.042	0.325	0.502
5.3	23.4	21.2	−0.138	0.145	0.459
Average					+0.482
			Potassium dichromate.*		
2.0	22.6	20.6	+0.150	+0.433	+0.552
3.0	22.8	20.6	0.083	0.366	0.543
4.0	21.8	19.7	0.041	0.324	0.550
5.3					
Average					+0.548
			Iodine.†		
2.0	19.8	17.9	+0.062	+0.345	+0.404
3.0	20.0	18.1	0.031	0.314	0.401
4.0	25.5	23.0	0.006	0.289	0.407
5.3	12.8	11.6	−0.014	0.268	0.422
7.0	25.5	23.0	0.045	0.238	0.441
Average					+0.415

* E_0 is here calculated where slope = 0.0592.

† E_0 is here calculated where slope = 0.0296.

In the case of iodine, constant values of E_0 were not obtained with this equation. The slope of the graph of E_0' in this case was one-half of what was expected from the equation, according to which the slope should be determined by the factor 0.0592. If, however, the slope is halved, Equation 10 becomes

$$E_0 = E_0' + 0.0296 \text{ pH} \quad (11)$$

When the data of the iodine titration are calculated by means of this equation, a constant value of E_0 is obtained, especially at the lower pH values (Table I). Just what the significance of this calculation is cannot be definitely stated. We have used the end-point of the titration as representing an oxidation which is 90.5 per cent complete, and this figure was derived on the assumption that two electrons were involved in the chemical change. The constancy of the E_0 values obtained in this manner from iodine titrations of both cysteine and thioglycollic acid is, nevertheless, very satisfactory.

The following tabulation gives a comparison of the values of the slope of the curve and E_0 for the three reagents used to oxidize cysteine.

Reagent.	E_0	Value of slope.
I_2	+0.415	0.0296
KIO_3	0.483	0.0592
$K_2Cr_2O_7$	0.548	0.0592

CONCLUSIONS.

Cysteine, prepared by the electrolytic reduction of cystine, was easily oxidized by the three reagents used, iodine, potassium iodate, and potassium dichromate. When so oxidized by titration methods, the curves showing the variation of potential as the oxidation proceeded were typical of the ordinary reversible oxidation-reduction system. These curves are quite similar to those obtained by Clark[9] for other systems.

Calculations of E_0, while checking within the limits of error for each reaction studied, showed somewhat different values for E_0 for each reagent used, indicating a difference in the reaction of cysteine with the various oxidizing agents. The values of E_0 were most satisfactory in the lower pH range and showed divergence as the pH increased above 5.3. These results may be

[9] Clark, W. M., and Cohen, B., *Pub. Health Rep., U. S. P. H. S.*, **37**, 933 (Reprint 834) (1923). Sullivan, M. X., Cohen, B., and Clark, W. M., *Pub. Health Rep., U. S. P. H. S.*, **38**, 1669 (Reprint 848) (1923).

considered as evidence for more than one oxidation product of cysteine.

As the end-products of the oxidations were not identified, the actual reactions are not as yet known. The products of the oxidations, while differing from each other, may or may not form with cysteine a reversible oxidation-reduction system of the usual type. The data of this article are insufficient to be of assistance concerning this point. That different values of E_0, indicating different reactions with the three reagents, should

TABLE II.

Thioglycollic Acid.

pH	Point of 90.5 per cent oxidation.	E_0'	E_0' (corrected).	E_0
		Iodine.*		
3.0	13.1	+0.044	+0.326	+0.415
5.2	13.9	+0.018	0.300	0.404
6.1	27.9	−0.040	0.242	0.422
7.25	12.1	−0.080	0.202	0.416
		Potassium permanganate.†		
3.0	15.5	+0.050	+0.332	+0.509
		Molecular oxygen.*		
6.1		−0.026	+0.256	+0.436

* E_0 is here calculated where slope = 0.0296.

† E_0 is here calculated where slope = 0.0592.

not be entirely unexpected is suggested by the various possible oxidation products of mercaptan groups, depending on the conditions and nature of the reagent. Such oxidation products include disulfides, sulfoxides, sulfones, and sulfonic acids. The formation of "intermediate" products in the oxidation of cysteine would seem to be in agreement with our results, because if the titrated solutions are allowed to stand for 24 hours a white crystalline substance which appears to be cystine separates out.

It is evident from Table II that quite similar remarks concerning the oxidation of thioglycollic acid could be made.

Finally, it has been suggested that the potentials observed by us depend not only upon the presence of cysteine and hydrogen ions but also upon the presence of an intermediate oxidation product of the cysteine in the solution. This appears to be a natural explanation for the fact that oxidation curves of normal type are obtained when a solution of cysteine is titrated with the common oxidizing solutions. The intermediate oxidation product is sufficiently stable to give reproducible potentials over a period of several hours in the cell which has been used.

STUDIES ON THE PHYSIOLOGY OF PYRIMIDINES.

II. THE METABOLISM OF THE NUCLEOSIDES OF URACIL AND CYTOSINE.*

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(*From the Division of Biochemistry, University of California Medical School, Berkeley.*)

(Received for publication, April 19, 1930.)

In a previous paper one of us (1) reported the results of an investigation dealing with the metabolism of certain pyrimidines. Those experiments showed that in the organism of the dog uracil and thymine are metabolized to yield urea. Cytosine, on the other hand, when fed to dogs, was partly excreted unchanged, partly deaminized to uracil.

Several questions presented themselves after completion of that investigation. How are pyrimidines metabolized when fed in the form of nucleosides? Is cytosine better utilized when it is combined with ribose? What is the behavior of the pentose present in the nucleosides? Is it utilized or is it excreted unchanged? The present investigation was undertaken in an attempt to find the answers for some of these questions.

There is only one investigation on record dealing with the metabolism of the pyrimidine nucleosides. Wilson (2) investigated the metabolism of uridine in the rabbit and in man. He concluded from his experiments that uracil nucleoside was destroyed in the body and not eliminated as such. He assumed that this destruction goes along two paths: In one case the pyrimidine is set free and is excreted as such; in the other case the pyrimidine ring is changed before the sugar is split off, so that it may be completely metabolized in the body. Wilson observed after feeding uridine in every experiment but one a large increase in the elimination of

* This investigation has been aided by a grant from the Board of Research of the University of California.

urea. He ascribed this rise in urea output to a slight toxic action of the uracil nucleoside, causing an increase in protein metabolism.

EXPERIMENTAL.

Female dogs were used exclusively in the experiments described below. The animals were kept on the standard diet described by Cowgill (3), except that the required amount of fat mixture was added to the food just before feeding. The plan of the experiments was to follow the various urinary constituents until a nitrogen equilibrium had been reached, to give the substance to be tested mixed with the food, then to continue the experiment until the output of these excretory products had returned to normal. The dogs were kept in metabolism cages, and the urine collected by catheterization every 24 hours. The animals were allowed to drink water *ad libitum.*

The following methods for analysis were used: total N, Kjeldahl method; urea, Van Slyke's gasometric method; ammonia, Folin's permutit method; inorganic sulfur, precipitation with benzidine and titration according to Drummond; pentose, Youngburg's method. Carbon determinations were carried out according to Friedemann and Kendall (4), but the apparatus was essentially that of Hibbard (5), except that the carbon dioxide was absorbed in ascarite and determined gravimetrically. For the isolation of the pyrimidines from the urine the method of Mendel and Myers (6) was used. As a test for the presence of uracil and cytosine in the urine we used the color reaction of Wheeler and Johnson (7).

Preparation of Cytidine and Uridine.

We prepared these compounds according to the methods of Levene and La Forge (8) and Levene and Jacobs (9), except that we modified their procedure for the preparation of cytidine. Instead of converting the cytidine nitrate into cytidine sulfate by way of the picrate, we found that flavianic acid (1-naphthol-2, 4-dinitro-7-sulfonic acid) is more satisfactory than picric acid for decomposing cytidine nitrate. The flavianate is easily decomposed by dissolving it in 5 per cent H_2SO_4 at 60° and extracting the flavianic acid with butyl alcohol from the warm solution, ac-

cording to Pratt (10). The cytidine sulfate obtained from the flavianate was extremely pure, and one recrystallization proved sufficient to obtain the base in beautiful crystals.

TABLE I.

Experiments with Uridine.

Dog A; weight 11 kilos.

	Date.	Volume of urine.	Total N.	Urea N.	NH_3N	Rest N.	Inorganic S.
		cc.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Experiment 1.	Mar. 28	175	5.53	4.36	0.342	0.83	0.298
	" 29	190	5.35	4.30	0.342	0.71	0.294
	" 30	195	5.42	4.30	0.350	0.77	0.293*
	" 31	185	6.03	4.86	0.286	0.88	0.270
	Apr. 1	170	5.56	4.53	0.325	0.71	0.292
	" 2	175	5.53	4.42	0.312	0.80	0.290
	" 3	180	5.60	4.56	0.311	0.73	0.294
Experiment 2.	Apr. 16	210	5.93	4.78	0.645†	0.51	0.320
	" 17	200	5.90	4.85	0.540	0.51	0.308
	" 18	210	5.91	4.80	0.625	0.49	0.314*
	" 19	200	6.35	5.18	0.453	0.72	0.295‡
	" 20	195	5.81	4.68	0.578	0.55	0.309
	" 21	215	6.13	4.98	0.625	0.53	0.321
	" 22	200	5.63	4.46	0.613	0.56	0.320

* Fed 5 gm. of uridine = 0.57 gm. of N.
† Determined on the following day.
‡ Wheeler and Johnson reaction, weakly positive.

An analysis of our compounds yielded the following results.

Cytidine. Found (Kjeldahl). N 17.28, 17.31.
Calculated for $C_9H_{13}N_3O_5$. N 17.31.

Uridine. Found. Sample A (Kjeldahl). N 11.40, 11.41.
" B " " 11.32.
" C " " 11.38, 11.36.
Calculated for $C_9H_{12}N_2O_6$. " 11.50.

Experiments with Uridine.

In Table I are recorded the results obtained with uridine. In Experiment 1 5.0 gm. of uridine mixed with the food were given to Dog A. The data show that the uridine was completely broken

down to urea. In Experiment 2 we find also an increased output of urea after feeding the compound. In this case we were able to detect a small amount of free uracil in the urine collected the day after the feeding. An examination of the data obtained in these two experiments shows a decrease in the output of inorganic sulfur after feeding uridine.[1]

In Experiment 3 (Table II) carbon and furfural were determined. To make Table II more complete we have added the

TABLE II.

Experiments with Uridine.

Dog C; weight 10 kilos.

	Date.		Volume.	Total N.	Urea N.	NH_3 N	Rest N.	C	Urea C.	Rest C.	C:N	Inorganic S.	Furfural.
			cc.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*		*gm.*	*mg.*
Experiment 3.	July	5	115	5.22	4.30	0.50	0.42					0.262	
	"	6	110	5.30	4.45	0.48	0.37					0.287	
	"	9	110	5.21	4.35	0.46	0.40	3.20	1.86	1.34	0.61	0.276	34
	"	10	110	5.25	4.42	0.41	0.42	3.27	1.89	1.38	0.62	0.283	37*
	"	11	120	5.75	4.63	0.50	0.62	3.75	1.98	1.71	0.65	0.282	61†
	"	12	115	5.51	4.60	0.51	0.40	3.55	1.97	1.58	0.64	0.270	28
	"	13	130	5.58	4.68	0.50	0.40	3.39	2.01	1.38	0.61	0.301	
Experiment 4.	July	14	110	5.18	4.30	0.45	0.43	3.38	1.84	1.54	0.65	0.280	
	"	15	110	5.21	4.35	0.45	0.41	3.25	1.86	1.39	0.62	0.278‡	
	"	16	110	5.65	4.60	0.43	0.62	4.16	1.97	2.19	0.74	0.248	
	"	17	95	4.95	4.21	0.38	0.36	3.15	1.81	1.34	0.64	0.260	

* Fed 5 gm. of uridine.

† Decided Wheeler and Johnson test.

‡ Injected 5 gm. of uridine in 15 cc. of water.

values for undetermined nitrogen, urea carbon, and undetermined carbon. Following the feeding of 5 gm. of uridine to Dog C we found an increase of 24 mg. of furfural in the urine. If this all came from free ribose, it would indicate 48 mg. of this sugar, according to Youngburg's tables. We tested for pentoses with Bial's reagent, and found the tests negative. A number of substances other than pentoses yield furfural under the conditions of Youngburg's procedure, especially glycuronic acid which yields

[1] A similar phenomenon has been observed after feeding isobarbituric and isodialuric acids to dogs (11).

about 10 per cent of its weight of furfural. We found (Table III) that under the conditions specified, cytidine and uridine yield about 17 per cent of their weight of furfural, so that if the total increased furfural production resulted from undecomposed nucleoside, it would represent only about 141 mg. of that substance in the urine. This is in reality a very small amount, considering that we were giving these substances in quantities far above the physiological level. We observed in every case after feeding uridine and cytidine an increase in the furfural-producing bodies

TABLE III.

*Furfural Production by Uridine and Cytidine under Conditions of Youngburg's Procedure.**

mg.		*cc.*			*mg.*			*per cent*
10	uridine	in 5	H_2O	produce	1.7	furfural;	production	17
2	"	" 1	"	"	0.39	"	"	19.5
2	cytidine	" 1	"	"	0.43	"	"	21.5
2	"	" 1	"	"	0.46	"	"	23
Blank.		2	urine	"	0.25	"	0.125 mg. per cc.	
2	uridine	in 1	"	"	0.43	"	production	15
2	"	" 1	"	"	0.50	"	"	19
2	cytidine	" 1	"	"	0.50	"	"	19
2	"	" 1	"	"	0.50	"	"	19

* The work reported in Table III was done in the chemical laboratories of the University of Hawaii and The Queen's Hospital, Honolulu. I wish to express my thanks to Professor Richard Wrenshall and Dr. Nils Paul Larsen for their courtesy.—O. H. E.

in the urine, amounting, however, never to more than 75 mg. of furfural.

In feeding experiments of this kind one has always to reckon with the possibility that the substances may be acted upon by the bacteria of the intestines. To test this possibility we carried out one experiment in which the uridine was injected. The data recorded in Table II (Experiment 4) show that the results were practically the same as those obtained after feeding the uridine.

Experiments with Cytidine.

In the first experiment (Experiment 5, Table IV) 4.0 gm. of cytidine mixed with the food were given to Dog A. As seen from

Table IV there is a marked increase in the output of urea in the urine, but coincident with this rise in urea we find an increase of all the urinary constituents determined. Evidently we are dealing here with a stimulation of cellular processes due to the ingestion of cytidine, which lasts for several days after the feeding of the substance.

In a second experiment (Experiment 6, Table IV) we fed to Dog D 2.70 gm. of cytidine. We observed in this case also a rise in the

TABLE IV.

Experiments with Cytidine.

	Date.	Volume.	Total N.	Urea N.	NH_3 N	Rest N.	C	Urea C.	Rest C.	C:N	Inorganic S.
		cc.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*		*gm.*
Experiment 5.	June 7	165	5.12	4.03	0.55	0.54					0.268
Dog A;	" 8	170	5.06	4.00	0.54	0.52					0.265
weight 11.2	" 9	170	5.10	4.04	0.52	0.54	3.40	1.73	1.67	0.67	0.267*
kilos.	" 10	210	5.84	4.55	0.61	0.68	4.15	1.95	2.20	0.71	0.284†
	" 11	200	5.55	4.47	0.58	0.50	3.67	1.92	1.75	0.66	0.295
	" 12	200	5.45	4.42	0.67	0.36					0.292
	" 13	210	5.71	4.63	0.62	0.46					0.316
Experiment 6.	July 10	150	5.24	4.24	0.47	0.53					0.274
Dog D;	" 11	155	5.33	4.13	0.60	0.60					0.306
weight 11	" 12	150	5.28	4.12	0.63	0.53	3.62	1.77	1.85	0.68	0.302‡
kilos.	" 13	150	5.55	4.35	0.51	0.69	3.62	1.86	1.76	0.65	0.284§
	" 14	200	5.70	4.57	0.62	0.51	3.66	1.96	1.70	0.64	0.290
	" 15	140	5.36	4.32	0.56	0.48	3.50	1.85	1.65	0.65	0.277

* Fed 4 gm. of cytidine; N = 0.69, C = 1.78 gm.

† Furfural in urine, 90 mg. Wheeler and Johnson test negative.

‡ Fed 2.70 gm. of cytidine; N = 0.47 gm.

§ Unable to get positive Wheeler and Johnson test.

total nitrogen and urea. The values for inorganic sulfur in the urine show a decrease following the ingestion of the nucleoside. Evidently in this case we are not dealing with an increased metabolism after the feeding of cytidine. It is reasonable to assume that in this case the compound was metabolized over a period lasting more than 24 hours.

Although in another experiment (Experiment 9, Table V) we did not observe any stimulating action of cytidine on the metab-

TABLE V.

Experiments with Uridine and Cytidine.

Dog B; weight 9.9 kilos.

Experiment No.	Date.	Volume.	Total N.	Urea N.	NH_3 N	Creatinine.*	Rest N.	C	Urea C.	Rest C.	C:N	Inorganic S.	Furfural.
	June	*cc.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*		*gm.*	*mg.*
7	13	130	4.81	3.96	0.47		0.30					0.280	
	14	130	4.75	3.89	0.47		0.31					0.279	
	15	130	4.62	3.80	0.46	0.200	0.28	3.03	1.63	1.31	0.66	0.263	28†
	16	130	5.35	4.26	0.51	0.215	0.50	4.24	1.83	2.32	0.79	0.279	85‡
	17	125	5.00	4.06	0.44	0.206	0.42	3.18	1.74	1.35	0.64	0.272	27
	18	135	4.83	3.81	0.48	0.206	0.46	3.14	1.63	1.42	0.65	0.274	21
8	19	125	4.65	3.73	0.46	0.206	0.38	3.06	1.60	1.37	0.66	0.252	
	20	125	4.78	3.90	0.43	0.206	0.37	3.09	1.67	1.43	0.65	0.259	29†
	21	130	5.26	4.18	0.43	0.198	0.57	3.68	1.79	1.81	0.70	0.254	68§
	22	120	4.48	3.62	0.38	0.198	0.40	2.92	1.55	1.29	0.65	0.237	23
9	23	125	4.45	3.57	0.51	0.206	0.29	2.98	1.53	1.36	0.67	0.230	
	24	120	4.36	3.49	0.43	0.235	0.36	2.96	1.50	1.37	0.68	0.232	||
	25	155	5.42	4.45	0.45	0.215	0.44	4.08	1.91	2.09	0.75	0.223	75¶
	26	125	4.36	3.50	0.46	0.206	0.32	2.92	1.50	1.34	0.67	0.223	
10	27	140	4.39	3.47	0.47	0.198	0.37	2.80	1.49	1.23	0.64	0.247	46||
	28	125	4.77	3.84	0.42	0.198	0.43	3.28	1.65	1.55	0.69	0.252	82¶
	29	130	5.00	4.02	0.45		0.45	3.00	1.72	1.20	0.60	0.262	22
	30	120	4.97	4.02	0.50		0.37	3.03	1.72	1.23	0.61	0.263	29
	July												
	1	110	4.66	3.76	0.43		0.39	3.00	1.61	1.31	0.64	0.262	

* We wish to express our thanks to Mr. O. L. Gericke for these determinations of creatinine. When creatinine was not determined, the value of 0.200 was assumed for calculating the rest N and rest C.

† Fed 5 gm. uridine; N = 0.57 gm., C = 2.21 gm.

‡ Strong Wheeler and Johnson test; recovered 80 mg. of uracil from one-third of the urine.

§ Wheeler and Johnson test positive, but weaker than on June 16.

|| Fed 4 gm. of cytidine; N = 0.69 gm., C = 1.78 gm.

¶ Decided Wheeler and Johnson test only when the urine was heated with bromine; indicates free cytosine rather than uracil.

olism, we believe that this compound, fed in doses of 4.0 to 5.0 gm. to dogs, causes an increased activity of cellular processes.

In Table V we present the results of a metabolic experiment lasting 21 days, in which total nitrogen, urea nitrogen, ammonia nitrogen, creatinine, carbon, inorganic sulfur, and furfural were determined. The data show that under the experimental conditions observed in this investigation the animal was in a constant nitrogen equilibrium for this length of time.

The results are practically the same as those recorded in Tables I to IV, except that the stimulating effect of cytidine was not observed in one of these experiments (Experiment 9, Table V).

The urinary carbon determinations were of value as a check on the observations of changes in the nitrogen distribution. An examination of the figures for rest carbon in Experiment 7 (Table V) shows an increase of 1.01 gm. after feeding uridine. In Experiment 9 we find an increase of 0.72 gm. after the ingestion of cytidine. This is far in excess of that which could arise from the amount of free pyrimidine plus undecomposed nucleoside which could be present as estimated either by the furfural values, or by the rest nitrogen. Apparently in these two instances the feeding of uridine and cytidine was followed by the excretion of a carbon-rich, nitrogen-poor substance. We are not in a position to state what this represents.

DISCUSSION.

The experiments described in this report show that uridine and cytidine, when fed to dogs, are broken down to urea. In a previous investigation it was found that cytosine was excreted partly unchanged, partly as uracil. The cytosine nucleoside, therefore, shows a behavior different from that of the free base. We find a similar phenomenon in the case of the purine derivatives of yeast nucleic acid. Severin (12) has shown that in man the nucleosides of the amino-purines, guanosine and adenosine, are metabolized to a greater extent than the free bases guanine and adenine.

The increase in undetermined N in some of our experiments seems to be definitely in excess of the undecomposed nucleoside plus free pyrimidine present. Steudel (13) attempted to show that pyrimidines may be converted into purines in the animal body, but his experiments were inconclusive.

Wilson (2) concludes from the data obtained after feeding uridine to man that this substance does not affect the output of uric acid or creatinine. It would have been interesting to determine whether the feeding of our compounds led to an increased elimination of allantoin, but unfortunately there is no very satisfactory method for determining allantoin. The observations of Ringer and Underhill (14) as to the effects of injections of yeast nucleic acid into the circulation of the fasting dog are of interest in this connection. These workers concluded from their experiments that yeast nucleic acid under those conditions induces a destruction of body tissue, giving rise to an increased output of nitrogen, creatine, and phosphates.

In Tables II, IV, and V we have included the values for the carbon to nitrogen ratio in the urine. Bickel (15) has called attention to the constancy of this quotient in man when the diet is controlled. Under normal conditions the value is, according to Bickel, 0.59 to 0.85. Schimizu (16) reports that the average C:N ratio for the dog partaking of a mixed diet is 0.68. Kauffmann-Cosla and Roche (17), working also with dogs, found it to be 0.74. Under the conditions of our experiments we observed an average C:N ratio of 0.65. This proportion of carbon to nitrogen in the urine remained practically constant from day to day. In seven out of eight experiments we obtained a rise in the ratio after feeding uridine and cytidine.

SUMMARY.

Experiments are described in which the pyrimidine nucleosides, uridine and cytidine, were fed to dogs maintained on a nitrogen equilibrium. Evidence was obtained that these compounds, when fed in small amounts to dogs, are metabolized to yield urea. The behavior of the nucleoside cytidine is in marked contrast to the free base cytosine, which is partly excreted unchanged, partly deaminized to uracil. Cytidine, fed in doses of 4.0 to 5.0 gm. to dogs, seems to stimulate the metabolic processes of the cells.

Ribose, the pentose sugar in the nucleoside molecule, is practically completely burned; certainly not more than a trace appears in the urine.

The average carbon to nitrogen ratio in the urine of dogs under

the experimental conditions observed in the present investigation was found to be 0.65. This quotient remained practically constant from day to day. In seven out of eight experiments there was noted a rise in the ratio after feeding uridine and cytidine.

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A SIMPLE INSTRUMENT FOR MICRO MANIPULATIONS.

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The instrument shown below is an inexpensive substitute for the much more perfect and expensive apparatus of Barber or of Chambers. At intervals during more than 3 years it has been used for such instrumental manipulations as those involved in puncture of Bowman's capsule in the frog kidney, obstruction of the neck of the renal tubule by pressure with a finely pointed rod, insertion of electrodes into a renal tubule, etc., operations which are performed under the 25 mm. lenses of a binocular microscope, and in which the movement most to be controlled is a direct thrust in one plane. It is similar in principle to certain types of automatic lead pencils.

Fig. 1 is a scale drawing showing its construction. A is a piece of square brass tubing, $4\frac{3}{4} \times \frac{3}{8} \times \frac{3}{8}$ inches; thickness of wall, $\frac{3}{64}$ inch. B is smaller square brass tubing, chosen or milled so that it slides without play through A. Its dimensions are $4\frac{3}{4} \times \frac{9}{32} \times \frac{9}{32}$ inches; thickness of wall, $\frac{5}{64}$ inch. One side of this is milled out for its entire length. C is $\frac{1}{4}$ inch round brass tubing, 3 inches long, $\frac{1}{8}$ inch bore, having a screw-thread cut on the outside, thirty-two threads to the inch. It is soldered into the end of B. D is a nut with a large knurled head, $\frac{9}{16}$ inch long, threaded to fit C. Close to its smaller end is a groove around it, $\frac{3}{64}$ inch wide, $\frac{1}{32}$ inch deep. E is a guide made of sheet brass, $\frac{3}{64}$ inch thick, $\frac{5}{8}$ inch wide at its greatest width. It is bent at a right angle, one limb of which is shaped into a fork to conform exactly to the groove in D. The other has two screw holes by which it is screwed to the end of A.

When these parts are assembled as shown in Fig. 1, to and fro movement of B C through A is effected by rotating the nut, D.

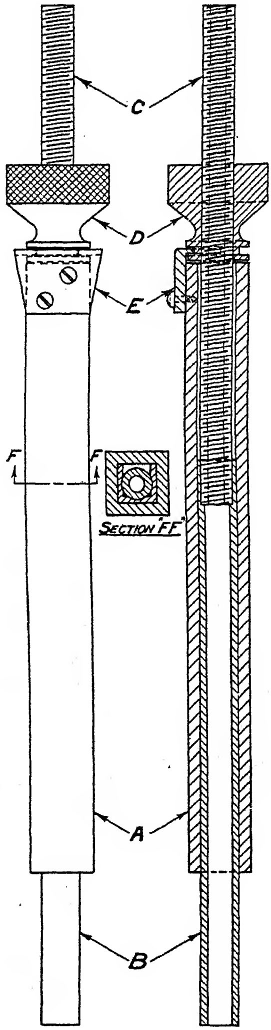

FIG. 1. Diagram showing construction of instrument for micro manipulations. An explanation of the letters is given in the text.

For collection of fluid from microscopic cavities or for micro injections, three-way stop-cock tubes are made of Pyrex tubing small enough to pass through the bore of the screw, with one arm

somewhat longer than the total length of the plunger, B C, with its screw. The long arm is thrust through the plunger so that the stop-cock is close against the end of the screw and fixed firmly in place by de Khotinsky cement applied at the ends and middle. Suitable tips for injecting or for compressing are cemented into the end of the long arm of the stop-cock tube.

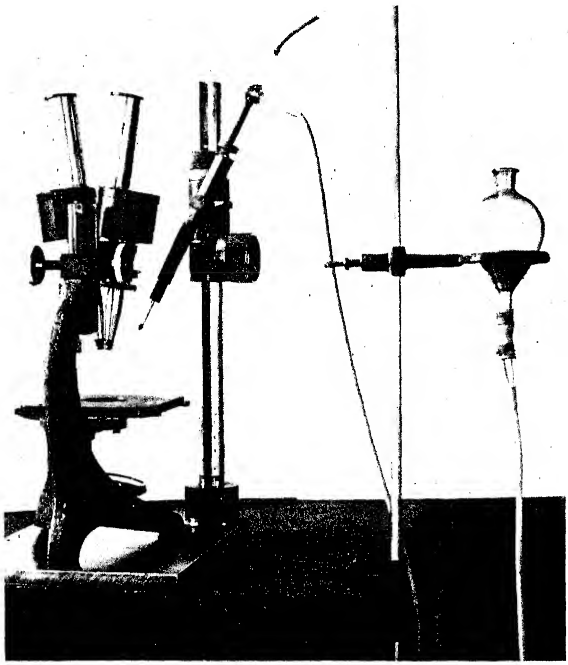

FIG 2. Micro manipulator mounted on Leitz support.

It is possible to use this instrument successfully for the purposes mentioned, when it is supported by an ordinary swivel clamp on the iron rod of a condenser support. Such a crude arrangement is not to be recommended. An exceedingly satisfactory support for the instrument can be made with the rack and pinion pillar from a Leitz binocular microscope support,[1] set into the corner of a rectangular piece of $\frac{1}{2}$ inch metal plate, $9\frac{1}{2} \times 7\frac{1}{2}$ inches, or into a

[1] Leitz Catalog V-A, 1929, stand GUG-42.

small heavy triangular casting, bored to take it (Fig. 2). The sleeve of the Leitz support which carries the arm for the microscope is removed and replaced by a similar sleeve to which is fixed a short swivel clamp. The rack and pinion gives easy and accurate vertical movement and one quickly learns to get any desired degree of horizontal adjustment by pushing or tapping the base of the microscope. This combination has served my purposes as well as has a Barber stage mounted on a Zimmerman stativ. When small heavy triangular bases for the rack and pinion post are available, several complete instruments can be grouped about one microscope.

No attempt is here made to advocate this instrument for tasks of such delicacy as those undertaken by Chambers and his pupils with the apparatus of his design.

A SECOND SERIES OF QUANTITATIVE ESTIMATIONS OF THE CONCENTRATION OF CHLORIDES IN GLOMERULAR URINE FROM FROGS.*

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(Received for publication, March 31, 1930.)

In 1925 Wearn and Richards (1) published the results of a short series of analyses which seemed to show that the concentration of Cl in the glomerular urine of frogs is significantly higher than in the blood plasma. They regarded the results as the "basis of reasonable doubt of the complete adequacy of the present conception of glomerular filtration" and stated, "If subsequent work shall confirm these, it is clear that the processes concerned in glomerular filtration require further study." The experiments summarized in this present paper were designed to amplify these data. The methods used were identical save in a few details which will be mentioned. A long preliminary period of practice was devoted not only to the nephelometric method of T. W. Richards for estimating extremely minute amounts of Cl but also to all the details of manipulation of the small volumes of fluid which are available in work of this nature.

Two groups of experiments were made: In the first the frog kidneys were perfused via the aorta with oxygenated modifications of Hamburger's solution.[1] Nineteen experiments showed identity

* Reported before The Thirteenth International Physiological Congress, at Boston, August, 1929 (*Am. J. Physiol.*, **90**, 277 (1929)).

The greater part of the analytical work referred to in this paper was done by Freeman; collections of blood and glomerular fluid were made largely by Livingston.

† National Research Council Fellow, 1924–26.

[1] Original formula, NaCl, 0.5; $CaCl_2$, 0.02; KCl, 0.01; $NaHCO_3$, 0.285 per cent. We reduced the $NaHCO_3$ to 0.2 per cent.

of Cl concentrations in fluid collected from Bowman's capsule and in the perfusing fluid. In the second series, glomerular fluid was collected from living frogs and its Cl content compared with that of plasma obtained by bleeding the animal at the end of the experiment. In this group are twenty experiments, in nineteen of which this comparison was made. In ten of these the figures for Cl concentration of the two fluids differed by less than 10 per cent, a difference which we regard as within the errors of the methods used. In nine the difference was more than this: in eight of these nine the glomerular fluid showed the higher concentration of Cl; in one of these eight, the difference was so little greater than 10 per cent as to be scarcely considerable. This leaves seven experiments out of twenty in which Cl of glomerular urine exceeded Cl of plasma by from 13 to 50 per cent.

At the time when these experiments were finished (July, 1926) they were the only ones with which the earlier analyses could be compared. In those, seven out of ten estimations indicated a power of the glomerular membrane to concentrate chlorides: in these, such indication is clearly seen in only seven out of twenty experiments; it is not seen in a series of perfusions of the surviving kidney. Clearly, these are not decisive and for that reason have not heretofore been published. In the three papers following this, new evidence on the question of the composition of glomerular urine is presented, obtained by different methods. Judged now, in the light of the new evidence, we are unable to see in these experiments any considerable weight of confirmation of the conclusion suggested by the results of Wearn and Richards. On the contrary, they agree better with the conclusion that the glomerular fluid is identical with a protein-free filtrate from plasma than with the conception of secretory enrichment of the glomerular fluid. Final decision on a question of so much difficulty can scarcely now be claimed. This publication is made so that those who are interested in the development of the subject may be in possession of all the results bearing upon it which seem competent enough to be worthy of consideration.

Methods.

Detailed description of technique of obtaining glomerular urine from frogs and of all the procedures involved in estimating its Cl

content by the nephelometric method of T. W. Richards will be found in the papers by Wearn and Richards (1, 2). Every precaution which they found necessary was scrupulously observed. The nephelometer was the same as that they used and the nephelometric estimations were made in the dust-free room described by them. The samples of glomerular urine in capillary weighing tubes were weighed on an assay balance sensitive to less than 0.01 mg.

The majority of frogs used were *Rana pipiens*; a few were *Rana catesbiana.* Urethane was used as an anesthetic in only four experiments; in all the others the brain and upper part of cord were destroyed by pithing.

In collecting glomerular urine the micro manipulator described in the preceding paper was used (3). In the perfusion experiments a cannula pointing toward the heart was inserted into the abdominal aorta just above its bifurcation. Perfusion flow began before the aortic ligature above the kidney at the level of the celiaco-mesenteric artery was tied. In the experiments on living frogs, maintenance of adequate circulation often required the introduction of fluid during the long period of glomerular urine collection. In the earlier experiments small quantities of 1 per cent glucose were repeatedly injected by a cannula in the anterior abdominal vein. In some experiments a urea solution was used; in one 0.4 per cent sodium chloride, and in a number at the end of the series a special solution containing 0.3 per cent NaCl, 0.15 per cent $NaHCO_3$, 0.04 per cent urea, and 0.1 per cent glucose. Separate experiments were made in an effort to judge the influence of such injections upon the NaCl content of the blood. Blood for estimation of Cl content of plasma was taken from the aorta at the conclusion of each experiment, and kept fluid by a minute amount of heparin. Weighed samples of plasma were analyzed for Cl by Whitehorn's method.

Results.

The results, expressed in terms of NaCl, are collected in Tables I to IV. Table I shows seven consecutive analyses of known solutions of pure sodium chloride by the nephelometric method. In each case a minute amount of the solution was taken into the capillary collecting pipette, discharged into a capillary weighing

TABLE I.

Consecutive Nephelometric Estimations of NaCl Concentration of Known Solutions Made during the Period Covered by Experiments of Table IV.

Date.	Weight of solution taken.	Concentration of solution.		Difference.
		Known.	Found.	
1926	*mg.*	*per cent*	*per cent*	*per cent*
June 15	2.16	0.42	0.43	+2.4
" 24	3.65	0.43	0.41	−4.7
" 30	1.23	0.40	0.42	+5.0
July 3	2.67	0.48	0.46	−4.2
" 19	2.02	0.40	0.38	−5.0
" 24	1.00	0.50	0.54	+8.0
" 27	1.13	0.44	0.47	+6.8
Average.......				+1.2

TABLE II.

Estimations of NaCl Content of Solutions Used in Frog Kidney Perfusions of Table III by the Whitehorn Method and by the Nephelometric Method.

Experiment No.*	Volume of solution used for Whitehorn estimation.	Weight of solution used for nephelometric estimation.	NaCl concentration as found by:		Difference.
			Whitehorn estimation.	Nephelometric estimation.	
	cc.	*mg.*	*per cent*	*per cent*	*per cent*
1	1	3.65	0.50	0.50	0.0
3	1	0.98	0.50	0.53	+6.0
4	1	2.88	0.50	0.54	+8.0
5	1	3.24	0.48	0.53	+10.4
6	1	3.39	0.51	0.53	+3.9
7	1	3.83	0.49	0.53	+8.2
8	1	1.45	0.51	0.48	−5.9
9	1	3.60	0.52	0.54	+3.8
10	1	2.00	0.49	0.52	+6.1
11	1	1.83	0.51	0.53	+3.9
12	1	4.44	0.48	0.48	0.0
14	1	3.01	0.51	0.48	−5.9
15	1	3.06	0.51	0.50	−2.0
18	1	0.69	0.25†	0.30†	+20.0†
19	1	4.32	0.24	0.24	0.0
Average.......			0.50	0.51	+2.6

* These numbers correspond to those in the first column of Table III.
† Not included in average.

TABLE III.

Perfusion Experiments. Chloride Content of Perfusion Fluid, Glomerular Fluid, and Bladder Fluid.

Experiment No.	Date	Time of preparation.	Perfusion pressure.	Glomerular fluid.			NaCl concentration in:			Difference between glomerular fluid and perfusion fluid.	Perfusion fluid.
				Collection time.	Collection pressure.	Amount.	Perfusion fluid.	Glomerular fluid.	Bladder fluid.		
	1926	*min.*	*cm. H_2O*	*min.*	*mm. Hg*	*mg.*	*per cent*	*per cent*	*per cent*	*per cent*	
1	Apr. 30		36	32	−30	3.79	0.50	0.46	0.18	−8.0	Hamburger's solution.
2	May 3	60	20	105	−30	2.37	0.50	0.54		+8.0	" "
3	" 4	70	26	45	−30	3.48	0.53	0.53	0.42	0.0	" "
4	" 5	120	16	45	−25	3.94	0.54	0.50	0.44	−7.4	" "
5	" 6	90	16	90		1.95	0.53	0.53	0.21	0.0	" "
6	" 7	300	18	33	−30	3.40	0.53	0.52	0.40	−1.9	" "
7	" 10	180	30–36	30		3.68	0.53	0.46	0.17	−13.2	" "
8	" 11	75	26	90	−100	1.48	0.48	0.48		0.0	" "
9	" 12	45	26	60	−70	1.19	0.54	0.61	0.37	+13.0	Same plus 0.04 per cent Na_2SO_4.
10	" 13	40	29	62	−45	1.94	0.52	0.46	0.21	−11.5	" " 0.04 " " "
11	" 14	50	40	40	−80	3.07	0.53	0.47		−11.3	" " 0.22 " " "
12	" 15	180	40	30		2.30	0.48	0.46	0.40	−4.2	" " 0.22 " " "
13	" 19	40	30	18	−75	1.83	0.48	0.53	0.25	+10.4	" " 0.4 " " "
14	" 19	45	30	55	−100	1.59	0.50	0.55	0.39	+10.0	" " 0.4 " " "
15	" 20	38	40	37	−80	1.88	0.26	0.33		+26.9	" " 0.47 " " "
16	" 24	70	40	45	−50	1.49	0.26	0.28		+7.7	" " 0.47 " " "
17	" 25	75	36	75	−60	0.89	0.25	0.24	0.02	−4.0	" " 0.47 " " "
18	" 26	50	39	75	−75	1.78	0.24	0.25	0.05	+4.2	" " 0.47 " " "
19	" 26	45	39	75	−75	1.94	0.24	0.25	0.13	+4.2	" " 0.47 " " "

TABLE IV.

Chloride Content of Plasma, Glomerular Urine, and Bladder Urine from Living Frogs.

Experiment No.	Date.	Weight of frog.	Glomerular urine.			NaCl concentration in:			Percentage difference between glomerular urine and plasma.	Notes.
			Collection time.	Collection pressure.	Amount.	Plasma.	Glomerular urine.	Bladder urine.		
	1920	*gm.*	*min.*	*mm. Hg*	*mg.*	*per cent*	*per cent*	*per cent*		
1	June 9	32	165	+15	1.88	0.30	0.30	0.04	0	Urethane, 0.6 cc. 25 per cent. Preparation, 2 hrs. 1 cc. 1 per cent glucose intravenously before and 1 cc. during collection of glomerular urine.
2	" 12	62	245	−45	0.44	0.32	0.29	0.07	−9.4	Urethane, 1.2 cc. 25 per cent. Preparation, 2 hrs., 40 min. 4 cc. 1 per cent glucose intravenously before and 2.8 cc. during collection.
3	" 15	57	180		0.54		0.27			Urethane, 1 cc. 25 per cent. Preparation, 2 hrs., 20 min. 1 cc. 1 per cent glucose intravenously before and 2 cc. during collection. Plasma lost.
4	" 16	70	150		0.39	0.32	0.30	0	−6.3	Urethane, 1 cc. 25 per cent. Preparation, 1 hr. 1.4 cc. 1 per cent glucose intravenously during collection.
5	" 19	55	185	−50	0.41	0.32	0.33	0	+3.1	Pithed. Preparation, 1 hr., 40 min. Circulation poor. 6 cc. 1 per cent glucose, 1 cc. 1 per cent urea intravenously during collection.
6	" 21	50	160	−50	1.87	0.36	0.31	0	−13.9	Pithed. Preparation, 1 hr., 40 min. 1.8 cc. 1 per cent glucose intravenously during collection.
7	" 22	52	180	−80	1.02	0.26	0.30		+15.4	Pithed. Preparation, $3\frac{1}{2}$ hrs. 2.4 cc. 1 per cent glucose intravenously before and 1 cc. glucose + 0.5 cc. 1 per cent urea during collection.
8	" 23	55	158	−35	1.33	0.38	0.55		+44.7	Pithed. Preparation, 45 min. 1 cc. 1 per cent glucose + 0.5 cc. 1 per cent urea intravenously before and 0.7 cc. glucose + 0.5 cc. urea during collection.

9	June 28	26	120	−75	0.90	0.31	0.30	0	−3.2	Pithed. Preparation, $1\frac{1}{4}$ hrs. 0.7 cc. 1 per cent glucose intravenously before and 2 cc. during collection.
10	July 3	215	90	+20	1.51	0.34	0.34		0	Pithed. Preparation, $1\frac{1}{4}$ hrs. 2 cc. 0.4 per cent NaCl intravenously before and 4 cc. during collection.
11	" 12	24	295		0.27	0.34	0.33	0	−2.9	Pithed. Preparation, 30 min. 2.5 cc. special solution subcutaneously during collection. Circulation slow.
12	" 13	25	55		1.06	0.39	0.46		+18	Pithed. Preparation, 1 hr., 40 min. 2.5 cc. special solution subcutaneously before and 2.2 cc. during collection.
13	" 15	25	100	0	1.35	0.47	0.43	0.06	−8.5	Pithed. Preparation, 1 hr., 10 min. 2.5 cc. special solution subcutaneously and 2.6 cc. intravenously before collection; 2.2 cc. intravenously during collection.
14	" 16	28	260		0.42	0.37	0.42	0	+13.5	Pithed. Preparation, 50 min. 2.5 cc. special solution subcutaneously before and 4 cc. intravenously during collection.
15	" 17	253	150		0.56	0.35	0.41	0.05	+17.1	Pithed. Preparation, 50 min. 1.5 cc. special solution intravenously before and 4 cc. intravenously during collection.
16	20	100	180	−150	1.75	0.42	0.47	0.008	+11.9	Pithed. Preparation, 1 hr., 55 min. 2.5 cc. special solution subcutaneously and 1 cc. intravenously before and 2 cc. intravenously during collection.
17	" 23	55	120	−60	1.44	0.43	0.52		+20.9	Pithed. Preparation, 50 min. 2.5 cc. special solution subcutaneously before and 1.2 cc. intravenously during collection.
18	" 24	73	80	−125	0.98	0.40	0.60	0.03	+50	Pithed. Preparation, 1 hr. 5 cc. special solution subcutaneously immediately after pithing.
19	" 26	49	120	−80	0.84	0.40	0.42		+5	Pithed. Preparation, 50 min. 5 cc. special solution subcutaneously immediately after pithing.
20	" 28	47	150	−28	1.69	0.40	0.38	0.03	−5	Pithed. Preparation, 70 min. 5 cc. special solution subcutaneously immediately after pithing.

tube, and weighed. Thus in all details except the collection of fluid from a Malpighian body these control estimations resemble those made on glomerular fluid. If the dates of these determinations are noted, it will be seen that they were made during the course of the experiments on living frogs summarized in Table IV. They therefore serve not only as evidence of the accuracy of the nephelometric method as used by us but also as periodic tests of the method during the time of its application to the fluid from frogs.

The greatest difference between Cl concentration found and Cl concentration known was 8 per cent of the latter; average difference was +1.2 per cent of the known concentration.

Table II contains fifteen comparisons of the nephelometric and the Whitehorn methods applied to the solutions used in perfusion experiments. In each case, 1 cc. was used for the Whitehorn estimation, and a minute weighed quantity for the nephelometric, taken as specified above. The differences in the last column are expressed as percentages of the value found by the Whitehorn method. Results by the two methods agree within less than 10 per cent in thirteen instances; in one the difference is slightly greater; in one the difference is 20 per cent.

The experiment numbers in the first column of Table II correspond with those in the first column of Table III.

Table III shows the results of nineteen perfusion experiments. The Cl concentration of the glomerular fluid is within 10 per cent of that of the perfusion fluid in thirteen experiments: the difference exceeds this in six. But in three of these the sign of the difference is +; in three −. Since both of the values compared were obtained by the nephelometric method it is not unreasonable to expect to find the dispersion of the differences somewhat greater in this group than in that of Table II. The results seem to us to show essential identity of the two fluids in respect of Cl content.

Table IV contains the results of twenty experiments on living frogs. All available information which bears on the validity of the results is included. In ten experiments the difference between glomerular urine Cl and plasma Cl is less than 10 per cent. We regard these as showing identity. In one of the others the Cl of glomerular urine is more than 10 per cent lower than that of plasma; in eight it is more than 10 per cent higher.

Three experiments were made to determine the influence on

plasma Cl of a voluminous subcutaneous injection of the "special solution" used in Experiments 11 to 20 of Table IV to maintain the circulation. In each, the frog was pithed, a cannula inserted into one arch of the aorta, and enough blood to yield 0.5 to 0.7 cc. of plasma taken. 5 cc. of the solution, which contained 0.3 per cent NaCl, were then injected under the skin of the thighs. The skin was kept moist with wet cloths and after from 50 to 60 minutes a second sample of blood was taken from the aorta. Cl was estimated according to Whitehorn.

Experiment No.	Weight of frog.	NaCl of first plasma.	NaCl of second plasma.
	gm.	*per cent*	*per cent*
1	54	0.42	0.40
2	43	0.41	0.39
3	44	0.45	0.37

The results indicate that plasma Cl may diminish slightly because of our injections; and since the blood sample was taken at the end of the period of glomerular collection, it may well be that in some of the experiments on living frogs (Table IV) the Cl content found was less than that of part of the plasma at least from which the glomerular urine was derived.

DISCUSSION.

The results of the perfusion experiments in Table III are not ambiguous. In them we see no reason to suspect that the fluid which emerges from the glomerular capillaries is anything else than a filtrate, its Cl content unmodified by the structures through which it has passed. That the perfused kidneys retained some, at least, of their vital properties is shown by the Cl content of the bladder fluid. In every sample tested this was lower than that of the perfusion fluid: in two instances the difference was of the same order as that encountered in the living frog.

The experiments on living frogs summarized in Table IV are the most important and at the same time the most difficult to interpret. Eight out of twenty experiments showed Cl content of glomerular fluid greater than that of plasma. In Wearn and Richards' experiments the ratio was seven out of ten. The question is

whether these seven experiments disclose a secretory power in the glomerular membrane capable of enriching the glomerular fluid in chlorides. In addition to the general criticism to which these experiments are open—*viz.*, that based upon the exacting technique which they demand—two specific criticisms may be brought forward. One concerns the Hg pressures in the collecting pipette system. In the majority of collections the mercury bulb was placed several cm. below the level of the kidney. Evidence, subsequently obtained in another connection, shows that it is possible to contaminate glomerular fluid with tubule contents. If such an error were present we should expect it to lessen the apparent Cl concentration of glomerular fluid because we know that chloride is reabsorbed. But knowledge of the part of the tubule in which preferential reabsorption of Cl takes place is still so scanty that the certainty of this expectation is doubtful.

The other specific criticism which may be offered concerns the possibility of evaporation of the glomerular fluid during the process of its transfer from collecting pipette to capillary tube in which it was weighed and in the brief time required to cap the latter. While this source of error has been recognized and guarded against from the very first days of this work in this laboratory, more recent experience arouses the suspicion that our precautions against it may not always have been completely successful. Obviously the likelihood of such an error would be greater, the higher the temperature at which the manipulations were carried out. The official temperatures in Philadelphia at 3 p.m. on the dates of four of the experiments in which Cl values of glomerular urine were found to be high (Experiments 12, 15, 17, and 18) were between 84 and 88°F.

This discussion indicates three reasons which may justify the belief that the present series of chloride estimations in glomerular urine does not confirm the suggestion given by the former series that the glomerulus can "secrete" chlorides. (1) Perfusion experiments show no indication of such capacity. (2) Evidence of its existence in living frogs was encountered much less frequently than in the earlier series. (3) Danger of concentration of glomerular fluid by evaporation after collection from living frogs may have been underestimated in both series.

SUMMARY.

Estimations of the Cl content of glomerular fluid from perfused frog kidneys show that it is the same as that of the perfusion fluid.

Similar estimations on glomerular urine from living frogs gave no striking confirmation of the earlier work by Wearn and Richards which suggested that the glomerulus may possess the power of "secreting" chlorides.

Notes Added during Proof Correction.—The reason for adding Na_2SO_4 to the perfusion fluid in some of the experiments of Table III was the thought that sulfate ions might hasten the movement of Cl through the glomerular membrane (*cf.* Goldschmidt and Dayton (4)). There is no clear evidence that it did. In Experiments 14 to 19, one-half of the NaCl of Hamburger's solution was replaced by 0.47 per cent Na_2SO_4. The designation of the perfusion fluid in these experiments is therefore not accurate.

The relatively great negative pressures used in collecting glomerular fluid in the majority of experiments may excite comment. It is not to be assumed that the pressures indicated by the level of the Hg bulb were transmitted directly and without loss to the contents of the capsule of Bowman. By way of explanation, it may be pointed out that the tip of the pipette projects into a *flowing* stream of fluid; furthermore experience has shown that the slightest obstruction at the tip of the pipette is extremely effective in impeding entrance of fluid into it and hence also transmission of the negative pressure.

Dr. L. V. Heilbrunn has suggested that the presence of glucose, urea, or other plasma constituents in the glomerular urine may be responsible for errors in the nephelometric comparisons.

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QUANTITATIVE STUDIES OF THE GLOMERULAR ELIMINATION OF PHENOL RED AND INDIGO CARMINE IN FROGS.*

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Evidence concerning the nature of glomerular function which has been derived from the comparative study of the composition of glomerular urine and blood plasma is not yet sufficiently concordant to yield decisive conclusion. Qualitative tests on material from the frog (1) and from *Necturus* (2) gave support to the theory of filtration by showing that certain blood constituents, most notably Cl and sugar, are eliminated from the glomerulus, only to be returned to the blood by the reabsorbing capacity of the tubule. One of two series of quantitative Cl estimations (3) furnished an indication of the existence in frogs of another glomerular process by which the concentration of Cl in the glomerular fluid is augmented; but a second series (4) gave results which were ambiguous, if not contradictory to those of the first. Experiments with *Necturus* suggested that phosphate in glomerular fluid is less concentrated than in plasma (5) while comparisons of total molecular concentration of plasma and glomerular urine appeared to show that the latter fluid is significantly hypertonic (6). The question therefore, in so far as it has been approached by these methods, whether filtration is the sole glomerular process or whether this is supplemented by something which we must call secretion, is still an open one.

The two chief obstacles in the way of further extension of evidence of this character are the technical difficulties in obtaining the fluid and handling it without loss or change, and the scarcity

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of chemical methods so refined as to be applicable to samples of material necessarily so small as are those available in this type of work. This paper contains an account of experiments in which the glomerular elimination of certain dyes was studied quantitatively, made possible by the discovery that satisfactory comparisons of color can be made in extremely minute amounts of solutions.[1] It is followed by two other studies, in one of which Barger's method of estimating total molecular concentration as used by White is applied to the glomerular urine of frogs and *Necturi;* in the other, success has been attained in determining the total electrolyte concentration (electrical conductivity) of glomerular urine from frogs and from *Necturi.* The work represented was continuous enough to be of uniform quality and possesses certain points of superiority over the earlier quantitative work from this laboratory on this subject, not only because of greater technical experience but also because of the incorporation of certain improvements into the technique. These include avoidance of negative pressure in collecting glomerular urine, a method for keeping the neck of the renal tubule obstructed during glomerular urine collection, and an easier method of collecting blood samples at any time during the experiment. Destruction of the brain by crushing instead of pithing removed one source of loss of blood; intravenous injections of fluid to maintain circulation have not been necessary.

It will be found that the results of the three studies are strikingly consistent and furnish strong support for the belief that the glomerular urine in frogs and *Necturi* has the composition of an ultrafiltrate of plasma.

Methods.

The following methods used in collecting glomerular urine, obstructing the neck of the renal tubule, and obtaining blood samples during glomerular urine collections were the same in the experiments of this and of the two papers which follow.

[1] The method (described on p. 485) was developed in experiments with Barnwell (1926) which were preliminary to those here presented. It promises usefulness beyond this particular field (see Went, S., and Drinker, C. K., *Am. J. Physiol.*, 88, 468 (1929) who utilized it in successive determinations of blood volume in small animals).

Collection of Glomerular Urine.—The frogs used (*Rana pipiens*) were exceptionally healthy specimens obtained from Vermont. The brain was crushed with a hemostat and the dissection made with a cautery, usually without any loss of blood. The skin over the abdomen was incised longitudinally from shoulder girdle to pelvis and transversely on the right side from the ventral to the dorsal mid-line. Two longitudinal incisions were made through the abdominal muscles, one on either side of the anterior abdominal vein, and these were connected by a transverse cut at the level of the ensiform cartilage. After dividing the anterior abdominal vein between ligatures the flap of muscle bearing the peripheral portion of the vein was reflected over the pelvis and a cannula inserted into it. The transverse incision through the abdominal muscles on the right side extended to the vertebral column. Other details of the preparation were similar to those described by Wearn and Richards. The surface of the kidney was not dried before inserting the pipette into a capsule of Bowman. It may be emphasized that the displacement of the kidney and tension on it in order to draw its lateral border over the window in the frog board is very slight, provided sufficient dissection along the vertebral column has been made and provided the body of the frog is properly adjusted in relation to the window. The utmost pains were taken never to touch the kidney itself and to keep it protected from exposure whenever it was not actually subjected to study.

The apparatus which one of us (W.) used for puncturing the capsules of Bowman and withdrawing glomerular urine was identical with that described by Wearn and Richards—a three-way glass stop-cock tube mounted on a Barber stage which gave movement in three planes, a finely pointed quartz tip, a levelling bulb and tube; the whole system was filled with mercury. For reasons of economy as well as preference, another of us (R.) preferred to use the simplified micro manipulator described elsewhere (7). We have found it advisable to puncture the capsule by repeated gentle thrusts rather than by one or two more vigorous efforts. The method of teasing an opening into the capsule is sometimes tedious but it involves less likelihood of making a hole so large that its edges do not adhere tightly to the outer surface of the quartz tip.

In the majority of experiments here described, the level at which the mercury bulb was placed during the collection of glo-

merular urine was the same as or higher than the level of the collecting point of the pipette after its insertion into a Malpighian body. Hence the flow of fluid into the pipette was the result of intracapsular pressure and not of suction. This provides added safeguard against collection of fluid which has entered the tubule.

Transfer of glomerular urine from the collecting pipette to the capillary tube in which it is to be further studied is accomplished as has previously been described (1). The time required for transfer and sealing of the capillary was usually about 1 minute.

Obstruction of Neck of Tubule.—Early in this study we discovered the desirability of blocking the neck of the tubule during a glomerular collection to avoid the possibility of contamination of glomerular fluid with fluid which was derived from or had passed into the tubule. Usually this was done by a method suggested by one of us and used by Hayman in his measurements of glomerular capillary pressure (8). A minute amount (about 0.5 c.mm.) of a solution of 0.5 per cent indigo carmine or 0.12 per cent phenol red in saline was drawn into the tip of the pipette before puncturing the capsule of Bowman. When insertion had been effected, this dye was allowed to flow slowly into the capsular space and from thence down the tubule which originated from it. This served to identify the single tubule which was to be blocked in the mass of tissue under observation. In the thin lateral portion of the kidney the tubule usually originates from the dorsal aspect of the Malpighian body and immediately turns directly or diagonally toward the extreme lateral border. The tissue is so thin that the tubule is usually easily visible when its lumen contains a colored fluid. After allowing all of the dye solution to flow into the capsule and on into the tubule, and after repeatedly drawing freshly formed glomerular fluid into the tip of the pipette and expelling it into the capsule, the fine, smoothly pointed tip of a glass or quartz rod was pressed against the surface of the kidney at a point directly above the neck of the tubule with sufficient force to close its lumen. The rod was manipulated in the same manner as was the collecting pipette, but from the opposite side of the microscope. In Tables III and IV describing the experiments this method is designated as "I. C. rod" or "P. R. rod" according to the dye used to identify the tubule.

We first used indigo carmine as the identifying dye. Thinking

that this might not be wholly without effect upon the glomerular membrane, we changed to phenol red. There is good reason for thinking that the latter could not damage the membrane with which it was in contact; but wishing to avoid any criticism which might arise because of the introduction of a foreign substance into the capsular space, we used two other methods in some of the experiments. One method (designated in Table III as "Hg") consisted in the injection of a minute globule of mercury into the intracapsular space in the hope that it would fall into the mouth of the tubule and so close it as long as there was positive pressure within the capsule. In a few instances this method was successful. The second alternative method consisted in making compression with the rod at a point near the punctured Malpighian body where, from experience, we expected that the neck of its tubule would be found. This is designated in Tables III and IV by the word "guess."

Whichever method was used, it was necessary to verify the correctness of the blockage at the end of the experiment. This has been done in two ways. (1) After the collected glomerular fluid had been transferred to a capillary tube and sealed, the blood circulation was stopped by excising the heart, the obstructing rod was lifted, a cannula inserted into the ureter, and this connected with a syringe filled with saline. The frog was then returned to the microscope stage, the obstructing rod replaced exactly as it had been during the experiment, and the piston of the syringe pressed. The fluid entered the tubules, driving the tubular contents back to the capsules of Bowman, distending them. When the obstructing rod was properly placed, the capsule from which collection had been made did not become distended. (2) After the collecting pipette had been emptied of glomerular urine, its tip was filled with a dye solution, reinserted into the capsule, and the dye injected. When the dye could be seen to stop at the obstructed point, the obstruction was regarded as having been verified.

When the dye method was used to identify the tubule before beginning glomerular collection, an observation of another sort was helpful in deciding whether obstruction had been effective. If the injected dye remained stationary throughout the period of collection in the middle portions of the tubule and became con-

centrated there, this showed that the stream of glomerular fluid had been prevented from flowing down the tubule.

Other Precautions.—In a considerable number of experiments it has been possible to see beyond any doubt that the tip of the pipette was sealed into the opening in the capsule of Bowman so that a completely tight joint was made. In some it was more difficult to be certain that a crevice did not exist which might have allowed contamination of the glomerular fluid with surface fluid. Hence in many experiments at frequent intervals during the glomerular collection a solution of a colloidal non-toxic dye of appropriate color (trypan blue or vital red) in salt solution was trickled over the surface of the kidney. The glomerular collection was carefully examined and when a trace of this color was found in it the experiment was discarded. This occurred only twice.

During the course of collection of glomerular urine it is necessary to watch the preparation almost constantly. The slightest movement of the frog may displace the tissue with relation to the tip of the pipette so that this slips out of the capsule or its tip impinges on the inner capsule wall so that collection ceases. It is advisable to test the correctness of the position of the tip as often as the slightest doubt arises. This is done by momentarily lifting the mercury leveling bulb a few mm. The resulting increase in intracapsular pressure causes the glomerular tuft to shrink. In the records of experiments, when the tuft is reported to be sensitive it is meant that it responds by visible change in size to very slight alterations in pressure.

The above details have been stated at length to indicate the fact that we have rigidly applied every precaution which we could think of not only to insure collection of glomerular fluid without contamination or change, but also to insure recognition of defects in technique when they occurred.

Collection of Blood.—A small cannula (capacity about 0.03 cc.) was inserted into the anterior abdominal vein pointing toward the pelvis. Before beginning glomerular collection it was carefully dried down to its extreme tip by slender rolls of absorbent cotton and a minute grain of heparin was deposited in the tip. Blood was prevented from issuing into it by rotating the cannula and so twisting the vein through one and one-half turns. When a sample of blood was desired during a glomerular collection, the

vein was untwisted. The first blood which flowed into the cannula was removed by inserting a glass capillary tube and was discarded: a second collection, similarly made and taken into a glass capillary, was kept. The ends of the capillary were sealed either in the flame or with plasticine and the tube immediately centrifuged. The lengths of the column of whole blood and of corpuscles were measured to give a hematocrit reading, and the tube cut so as to separate the plasma. The tube containing the plasma was sealed and laid aside for subsequent comparison with the glomerular fluid. The cannula was carefully cleaned between each two blood collections. Heparin for subsequent samples was introduced into the capillary receiving tube rather than into the cannula.

By this simple means as many samples of blood as were desired could be taken during the course of an experiment without touching the frog, frog board, or microscope. The volume of each sample was of the order of 0.02 to 0.03 cc., ample for our purposes.

Estimation of Concentration of Dye in Minute Volumes of Glomerular Urine and Plasma.—A supply of hard glass capillary tubes ranging in diameter from 0.2 to 0.5 mm. was procured from a glass-blower. Professional skill is needed in making them in order to avoid irregularities of bore. They were sorted according to inner diameter by holding them vertically with the lower ends immersed in a shallow dish of ether. Those in which the height of the ether column was the same had the same diameter.[2] Six or eight of the same bore were made ready for a single estimation.

As soon as collection of glomerular fluid from a Malpighian body was finished the fluid was transferred to one of these capillary tubes and drawn in a little way from the end.

The reaction of glomerular urine is not such as to develop the maximum color of the phenol red dissolved in it. Hence it was necessary to make the sample alkaline beyond the color range of the dye, without altering the volume of the sample. To do this the tube containing it was laid upon a wet cotton mat upon which a few drops of concentrated ammonia had been poured, and covered by an inverted beaker, the inner surface of which was wet

[2] We owe this bit of technique to the suggestion of the University glass-blower, Mr. J. D. Graham.

with water. In a few minutes the maximum color change was produced and then the tube was removed and sealed.

A series of standard dilutions of phenol red in test-tubes was prepared, enough alkali being contained in each to give maximum color. A range of from 1 to 20 mg. per 100 cc. has served our purposes. Capillary tubes of the same bore as that containing the glomerular urine were filled from these and the standard tube identified which most nearly matched the color in the glomerular urine.

Particular care is required in making the color comparisons. We have found it helpful to cut two parallel slits in a piece of white paper separated by a distance slightly less than the length of the column of the glomerular urine in the capillary tube. The tube is then pushed through these slits so that only that portion of the tube is visible which contains the colored fluid. In similar fashion, a tube of standard solution is placed on either side of this. They are examined in strong daylight, shadows being avoided. A reading glass is often helpful. Test experiments many times repeated showed that we could consistently identify the concentration of any of our standard solutions, used as an "unknown," in volume as small as 0.2 c.mm. The standard series was 1, 2, 3. . . up to 10, and 10, 12, 14 . . . up to 20 mg. per 100 cc.

Dye concentrations in blood plasma were determined in the same way, and the same procedures, omitting that for making the fluids alkaline, are applicable to the estimation of indigo carmine.

Concentration of Phenol Red in Ultrafiltrates of Serum and Plasma. —Small collodion (parlodion) sacs were made from a 6 per cent solution in equal parts of alcohol and ether. Before use they were thoroughly washed with water, partially dried with a current of air, and the inside rinsed with a portion of the fluid to be filtered. Filtration pressure was 100 to 150 mm. of Hg. Adequate precautions were taken to avoid dilution of the filtrate with water contained in the membrane or concentration of the filtrate by evaporation.

The following data were obtained in filtrations of fluids containing phenol red.

1. Five determinations were made of the concentration of phenol red in filtrates from Hamburger's solution containing 12 mg. per cent of phenol red (no protein). The lowest value obtained,

expressed as percentage of the concentration in the mother fluid, was 98.4 per cent; the highest, 100 per cent.

2. Sterile normal horse serum (Mulford) was diluted with equal volume of Hamburger's solution and phenol red added to make 12 mg. per cent. Six separate experiments gave the following values for phenol red concentration in the protein-free ultrafiltrates, expressed as percentages of the concentration in the mother fluid: 41.5, 40.7, 42.5, 41.8,[3] 40.0,[3] 39.3; average 41.0 per cent. In two other experiments, the fluid was allowed to stand in an ice box for 24 hours and 48 hours respectively, before filtration. Results, 50.0 and 53.4 per cent.

TABLE I.

Phenol Red Concentrations in Ultrafiltrates from Frog Plasma.

Date.	Source.	Red blood cell volume (percentage of blood volume).	Phenol red added to plasma to make mg. per cent.	Phenol red in filtrate (per cent of concentration in plasma).
1928				
Mar. 6	6 frogs.		14.6	66
Apr. 3	4 "	11	12	83
" 13	7 "	19	12	69
1929				
Apr. 10	2 frogs.	32	11	66
" 11	4 "	14	12	75
		13	13	84
		24	14	60
Apr. 12	2 frogs.	32	25	60

3. Four similar experiments were made in which normal horse serum, containing 0.35 per cent of phenol as preservative, diluted with equal parts of Hamburger's solution, was filtered. Results: 64.6, 68.8, 70.7, 68.7; average 68.1 per cent.

4. In six experiments, heparinized frog plasma containing phenol red was filtered, with results which are shown in Table I.

These results show that when sterile normal horse serum is diluted with Hamburger's fluid, phenol red added, and filtered through collodion the average concentration of dye in the protein-free filtrate is 41 per cent of that in the serum. Filtration of

[3] These filtrations were made through cellophane, an Aitken apparatus being used.

frog plasma containing phenol red gives far more divergent values, the variations being between 60 and 84 per cent. It will be noted that the three highest values for filtrates were obtained with plasmas having the three lowest hematocrit readings, and that the three lowest filtrate values came from plasmas having the three highest readings. This means that the variations are due, largely at any rate, to differences in degree of blood dilution; *i.e.*, concentration of plasma proteins. The lower hematocrit values resulted from the fact that in collecting blood from the aorta we sometimes injected saline solution under the skin and allowed it to be absorbed before finishing the blood collection.

Phenol Red Concentration in Glomerular Fluid from Perfused Kidneys.

In a number of instances the phenol red concentration in an ultrafiltrate from frog plasma containing it differed so little from that of the plasma as to make us fear that indecisive results would be obtained in experiments on living frogs. Although it was found later that this fear was not justified, in our first experiments the kidneys were perfused with a fluid made by diluting sterile normal horse serum with equal volume of Hamburger's fluid. Experiments by Drinker (9) have shown the advantages of horse serum as a constituent of frog perfusion fluid and the tests described above show the high capacity of horse serum proteins for adsorbing phenol red. From those results it would be expected that the concentration of color in glomerular fluid taken from kidneys perfused with diluted horse serum to which phenol red has been added would be about 40 per cent of that in the perfusion fluid itself, assuming that the glomerular fluid is a protein-free filtrate. The usual perfusion technique was used, pains being taken not to interrupt circulation through the kidney. Perfusion pressure varied between 23 and 35 cm. The perfusion fluid was a filtered, thoroughly oxygenated mixture of equal parts of horse serum and Hamburger's solution, to which phenol red was added to make 12 mg. per cent. In Experiment 1, a serum was inadvertently used which contained 0.35 per cent of phenol; in all the others, the serum contained no preservative. In Experiments 1 to 4, the tubule was unobstructed; in Experiments 5 and 6 it was obstructed

by pressure with a rod, after having been identified by intracapsular injection of indigo carmine. Adequacy of the obstruction to prevent reflux was verified at the end of the experiments by ureteral injection.

The results are shown in Table II. The column headed "Collection pressure" contains figures which show the height of the mercury leveling bulb in relation to the level of the kidney. The minus sign indicates negative pressure. The technical details of all of these experiments were performed rapidly and without mishap. Perfusion flow in Experiment 4 was scanty (constriction

TABLE II.

Perfusion of Frog Kidney with Diluted Horse Serum Containing 12 Mg. Per Cent of Phenol Red.

Experiment No.	Date.	Duration of glomerular collection.	Concentration of phenol red in glomerular fluid.	Collection pressure.	
	1928	*min.*	*mg. per 100 cc.*	*mm. Hg.*	
1	Feb. 8	42	12–13	*	Perfusion fluid contained 0.175 per cent of phenol. Tubule open.
2	" 13	43	24	*	Tubule open.
3	" 14	10	12	*	" "
4	" 18	32	20	−20	" "
5	Mar. 1	27	5	0	" blocked.
6	" 2	33	5	−60	" "

* Not recorded; believed to have been negative.

of arterioles?) so that an hour elapsed before glomerular collection began.

The results are striking. When the tubule was blocked, the concentration of phenol red in the glomerular fluid was exactly the same as that found in ultrafiltrates from the same perfusion fluid through collodion. In the four experiments (Experiments 1 to 4) in which the tubule was not blocked, the dye concentration was far higher than that in an ultrafiltrate; in two cases higher than in the perfusion fluid itself. In one of these the glomerular structures were subjected to 0.175 per cent phenol. This was the reason for thinking that the high values in the glomerular fluid

could not be due to a "secretory" activity of the glomeruli, and for the decision to block the tubules in subsequent experiments before beginning glomerular urine collection.

More direct evidence was secured that fluid from the capsule may enter the tubule, become concentrated there, and then be drawn into the capsule and taken into the pipette. Frog kidneys were perfused via the aorta with oxygenated Hamburger's solution containing phenol red. As soon as the tubules were filled with the fluid, aortic perfusion was interrupted for a few minutes (to give time for concentration), then resumed at lower pressure with Hamburger's solution containing *no* phenol red, and glomerular fluid collection with negative pressure begun. The collected fluid contained phenol red.

We conclude therefore that in respect of phenol red, the glomerular fluid in these experiments was an ultrafiltrate as shown in Experiments 5 and 6, and that the high values obtained in Experiments 1 to 4 were due to reflux into the capsule of some of the filtrate which had been concentrated within the tubule.

Phenol Red Concentration in Glomerular Urine from Living Frogs.— The phenol red was injected under the skin of the thighs, from 1 to $2\frac{1}{2}$ hours before beginning of glomerular collection, dissolved in 1 to 4 cc. of salt solution according to the weight of the frog. Often, though not always, it seemed advisable to inject a little more saline solution (1 to 2 cc.) subcutaneously during the dissection. In a few experiments, a small amount more was injected during the course of the glomerular urine collection through a glass tube which had previously been inserted through the skin of one thigh. These details are not included in the tabulation of the experiments since there is nothing to show that they had any influence on the results.

In five of the experiments urea was added to the saline solution injected (0.05 to 0.1 per cent) with the thought that it might encourage a "secretory" activity if such existed. In every experiment attempt was made to obstruct the neck of the tubule.

Altogether twenty experiments have been made. The description and results of fifteen of these are collected in Table III. Since perfection of technique is a matter of so much importance in these experiments, they are divided into two groups of nine and six respectively. Those in the first group were designated sa

"perfect" or "very good," meaning that every item in the performance was properly carried out. Those in the second group of six were, in one respect or another, not quite so satisfactory, but in no instance was the defect such as to throw serious doubt upon the approximate accuracy of the result.

The reasons for discarding five experiments, the results of which were not in accord with those of the other fifteen, are as follows:

Experiment 12.—The hole in the capsule was so large that fluid from the surface of the kidney entered the capsule and was taken into the pipette. The crevice could be seen and trypan blue on the kidney surface discolored the glomerular collection.

Experiments 20, 28, and 30.—Adequate blocking of the tubule could not be verified. Trouble was encountered in transferring the glomerular urine of Experiment 28 to capillary testing tube, hence there was a possibility of evaporation. The plasma phenol red concentrations in these three experiments were 7.3, 6, and 6 mg. per cent respectively; glomerular urine concentrations were 8, 9, and 22 mg. per cent.

Experiment 11.—This experiment technically was one of the best. We were unable to point out any known defect. It is impossible however to accept the result. Average concentration of phenol red in the plasma was 5.5 mg. per cent; in the fluid collected apparently from the glomerulus, 84 to 90 mg. per cent. A phenol red solution as strong as 0.09 per cent is so intensely colored that a capsule of Bowman, when filled with it, stands out like a red balloon from surrounding tissue. The fluid in the intracapsular space during collection was not visibly red. Hence we conclude that in some unaccountable way, the concentrated fluid from a neighboring or possibly a connecting tubule entered the pipette.

The results of the fifteen tabulated experiments seem to us not only to be consistent with, but to force the conclusion that in so far as phenol red is concerned, the glomerular urine is an ultrafiltrate. Ultrafiltration through collodion of frog plasma to which phenol red has been added gave a range of values of the filtrates from 60 to 84 per cent of the mother fluids. The range of these results on glomerular urines taken from living frogs is from 50 to 95 per cent; the range of twelve of the fifteen is between 56 and 87 per cent.

TABLE III.

Phenol Red in Glomerular Urine and Blood Plasma from Frogs.

Experiment No.	Date.	Weight and sex of frog.	Weight of dye injected.	Method of blocking tubule.	Glomerular urine. Collection pressure.	Glomerular urine. Time of collection.	Time of blood samples / R.B.C. volume.†	Concentration of phenol red in: Plasma.	Concentration of phenol red in: Glomerular urine.	Concentration of phenol red in: Glomerular urine.	Notes.
	1928	*gm.*	*mg.*		*mm. Hg*	*min.*	*min.* / *per cent*	*mg. per cent*	*mg. per cent*	*per cent of plasma phenol red*	
10	Mar. 24	55 ♀	3	I. C.;* rod; verified.	+30→+10	68	24, 39, 56	—, 4, —	3	75	
13	" 28	29 ♂	2	" "	+20→+10	50	4, 8, 46, 54	8, 8, 7, 7	5.7	76	T. B.* on kidney. Tuft retracted.
15	Apr. 3	34 ♂	2.4	" "		46	-2, 40, 53 **18, 17, 18**	—, 5, —	4	80	" "
14	Mar. 29	♂	1.8	I. C.; Hg; verified.	+10	46	6, 41, 55 **45, 47, 49**	14, 14, 14	8	57	" "
19	May 5	35 ♀	2.4	P. R.;* rod; verified.		60	−10, 5, 57, 67 **12, 13, —, 15**	9.5, 9.0, —, 6.5	7	84–90	Urea. Tuft retracted. Bladder urine = 151 mg. P. R. per cent.
	1929										
29	Apr. 2	72 ♀	3	P. R.; rod.	+3	30	10, 25	5, 4	3	67	Same frog as in Experiment 28, different glomerulus. Verification of block not attempted. Tuft retracted.

* The abbreviations I. C., P. R., and T. B. mean indigo carmine, phenol red, and trypan blue, respectively.

† In this column, "Time of blood samples" is expressed as minutes after (or before) the beginning of glomerular urine collection. Figures in bold-faced type are the hematocrit readings.

33	Apr. 5	57♀	3	Guess; verified.	+3	20	10 28	7	5	71	
34	" 5	57♀	3	" "	+3	35	8 38	5	4−	80−	
35	Mar. 12	54♀	3	P. R.; rod.	+3	46	14, 32 31, 30	12, 12−	8	67	
	1928										
9	Mar. 7	58♀	3	I. C.; Hg.; uncertain.		20	6		< plasma.		Could not see proximal tubules, hence Hg. Obstruction probably effective. Too little glomerular urine for quantitative estimation.
18	Apr. 24	25♂	1.8	I. C.; rod; uncertain.	0	45	10, 38, 52 22, 20, 28	4.5, 4.0, 4−	4(+?)	95	Urea. Obstruction probably effective. T. B. on kidney. Plasmas discolored, hence comparisons probably inaccurate. Bladder urine = 71 mg. P. R. per cent.
21	May 11	22♂	1.8	Guess; verified.	−20	43	−35, 50 8, 8	4.5, 4.1	3.7	86	Urea. Circulation poor. 1 cc. urea-saline solution intravenously before collection; 0.3 cc. during collection.

TABLE III—*Concluded.*

Experiment No.	Date.	Weight and sex of frog.	Weight of dye injected.	Method of blocking tubule.	Glomerular urine.		Time of blood samples / R.B.C. volume.†	Concentration of phenol red in:			Notes.
					Collection pressure.	Time of collection.		Plasma.	Glomerular urine.		
	1928	*gm.*	*mg.*		*mm. Hg*	*min.*	*min.* / *per cent*	*mg. per cent*	*mg. per cent*	*per cent of plasma phenol red*	
22	May 14	29♂	2.4	P. R.; rod; slightly questionable.	0	25	5, 17, 35 21, 24, 30	4, 4, 4	2	50	Urea. Long delay during operation. 4.9 cc. urea-saline subcutaneously during operation. Obstruction probably effective. T. B. on kidney.
	1929										
31	Apr. 4	45♀	3	Guess; slightly questionable.	+3	22	12 23	12	9	75	Hemorrhage during operation but circulation good. Tuft retracted. Obstruction probably effective.
32	" 4	45♀	3	Guess; slightly questionable.	+3	35	15 20	10	7	70	Same frog as in Experiment 31, different glomerulus. Tuft retracted. Obstruction probably effective.

There is no strict correlation between degree of blood dilution as shown by the hematocrit values of red blood cell volumes and the ratio of phenol red in glomerular urine to that in plasma; it will be seen however that four of the lowest hematocrit values are associated with the four highest ratios.

Experiment No.	22	29	14	32	33	100	31	13	34	15	21	19	18
Phenol red ratio	50	54	57	70	71	75	75	76	76	80	86	90	95
R. B. C. volume per cent.	24		47	20	28		23		38	18	8	13.5	24

In six experiments of this series, quantitative comparison was made between total molecular concentrations of glomerular urine and plasma by Barger's method. Details of the experiments are tabulated in the following paper by Walker. The phenol red concentrations of these glomerular urines varied between 67 and 76 per cent of the concentrations in the plasmas. In one, enough plasma was obtained for ultrafiltration: the plasma contained 12, and both the glomerular urine and the ultrafiltrate contained 8 mg. per cent. Comparison of the total molecular concentrations in five of the six showed identity within the errors of the methods: in one the glomerular urine appeared to be more concentrated than plasma, but this was one of a group in which the portion of glomerular urine taken for this determination was very small and in which therefore the result was probably influenced by evaporation.

Indigo Carmine.

The degree of adsorption of indigo carmine by the plasma proteins of frog blood is greater than is that of phenol red. In one experiment, plasma from the blood of four frogs, containing 10 mg. per cent of indigo carmine, was filtered through collodion and yielded a filtrate containing 3.9 mg. per cent. The red blood cell volume was 15.6 per cent. In another experiment, plasma of blood from three frogs, red blood cell volume 22.6 per cent, containing indigo carmine, 10 mg. per cent, yielded a filtrate containing 3.3 mg. per cent.

Five experiments only have been made with living frogs in which glomerular urine was collected during the elimination of subcutaneously injected indigo carmine. They are summarized

TABLE IV.

Indigo Carmine in Glomerular Urine and Blood Plasma from Frogs.

Experiment No.	Date.	Weight and sex of frog.	Weight of dye injected.	Method of blocking tubule.	Glomerular urine.		Time of blood samples / R.B.C. volume.†	Concentration of indigo carmine in:			Notes.
					Collection pressure.	Time of collection.		Plasma.	Glomerular urine.		
	1928	*gm.*	*mg.*		*mm. Hg*	*min.*	*min.* / *per cent*	*mg. per cent*	*mg. per cent*	*per cent of plasma I. C.**	
27	May 24	48 ♀	4	Guess; verified.	0	57	1, 16, 47, 52 **26, 25, 18, 22**	11+, 11, 11+, 11−	4	36	Urea. 0.5 cc. urea-saline subcutaneously during collection. V. R.* on kidney.
23	" 18	26 ♂	2	P. R.;* rod; verified.	−20→−10	55	3, 30, 49 **35, 27**	5+	3	60	Urea. 0.5 cc. urea-saline during collection.
26	" 23	40 ♀	4	P. R.; rod; verified.	−10	60	2, 22, 42, 55 **26, 24, 24, 23**	13, 12, −, 12−	6	50	Urea. V. R. on kidney.
24	" 19	35 ♂	4	P. R.; rod; not verified.	−10	44	3, 19, 37, 53 **28, 25, 26, 28**	9, 9, 9−, 9	4.5	50	Urea. 0.6 cc. urea-saline subcutaneously during collection.
25	" 21	28 ♂	4	P. R.; rod; uncertain.	0	61	3, 27, 52 **32, 40**	12.5, 12.5, 12.5	5	40	1 cc. saline subcutaneously during collection. Glomerular urine scanty. V. R. on kidney. Trace of red in glomerular urine. Color estimations not perfect.

* The abbreviations P. R., I. C., and V. R. mean phenol red, indigo carmine, and vital red, respectively.

† In this column, "Time of blood samples" is expressed as minutes after (or before) the beginning of glomerular urine collection. Figures in bold-faced type are the hematocrit readings.

in Table IV. The color of dilute indigo carmine solutions is not as advantageous for capillary tube colorimetry as that of phenol red solutions and for this reason we do not regard our color comparisons, particularly in plasma, as having the same accuracy as in the phenol red experiments.

The range of values of indigo carmine in glomerular urine, expressed as percentage of the value in the plasma (36 to 60), is somewhat higher than was found in the ultrafiltration experiments, described above. In view of what has just been said, we do not regard this as legitimate reason for believing that any other glomerular process was concerned in the glomerular elimination of indigo carmine in these experiments.

Possibly it should be said that in every experiment both with phenol red and with indigo carmine, direct color comparison was made between the glomerular urine and the plasma. In every instance the color of the glomerular urine was less intense than that of the plasma, the difference being obviously greater with indigo carmine than with phenol red. The magnitude of the color difference between plasma and glomerular urine is shown by our figures to be of the same order as that between similar plasma and an ultrafiltrate from it.

CONCLUSIONS.

Glomerular fluid collected from capsules of Bowman in the frog kidney may become contaminated with fluid drawn back from the tubule unless the precaution of obstructing the neck of the tubule is adopted.

When this precaution is taken the values of the concentrations of phenol red and indigo carmine in glomerular urine collected during the renal elimination of these dyes bear the same relation to their concentrations in the blood plasma as is obtained when similar plasma is filtered through collodion.

This study of the glomerular elimination of phenol red and indigo carmine gives no ground for belief in the existence of any other glomerular process than that of filtration.

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COMPARISONS OF TOTAL MOLECULAR CONCENTRATION OF GLOMERULAR URINE AND BLOOD PLASMA FROM THE FROG AND FROM NECTURUS.*

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In 1928 White (1) discovered the feasibility of utilizing Barger's microscopic method of estimating molecular weights for comparing the total molecular concentration of glomerular urine and blood plasma from the mud-puppy (*Necturus maculosus*). The method consists in measurement of change in volume of droplets of the two fluids when placed in juxtaposition in a capillary tube. White applied the method in a series of twelve experiments: in each case the concentration of the glomerular urine was found to be significantly higher than that of the plasma and the conclusion was drawn that the glomerular membrane is an actively secreting structure.

The same conclusion, more tentatively stated, had previously been suggested by the first quantitative studies made of glomerular urine from the frog; *viz.*, the chloride estimations by Wearn and Richards (2). But despite this fact it seemed wise to accept it with some reservations. A second series of Cl estimations had not furnished convincing confirmation of the results of the first (3). Estimations of the concentrations of phenol red in blood plasma and glomerular urine from frogs showed the same difference between the two fluids as that between the plasma and an ultrafiltrate from it, forcing the conclusion that in respect of the elimination of these dyes in the frog the glomerular urine is an ultrafiltrate (4). The possibility was also discovered of contaminating the glomerular fluid during its collection with fluid drawn back

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from the tubule, and measures adopted which prevented this. While it seemed unlikely that such contamination took place in White's experiments and, if it did, that it could have produced increase in concentration of the fluid taken from the capsule of Bowman, we were anxious to know whether White's experience with *Necturus* could be duplicated in similar experiments on the frog. Accordingly we made a study of Barger's method as used by White and have applied it in comparisons of the total molecular concentration of frog blood plasma and glomerular urine. The results of thirty-three experiments showed with convincing consistency that the concentrations of the two fluids were the same. In all but four of these experiments the tubule was closed during the glomerular collection to avoid the possibility of drawing fluid from the tubule into the collecting pipette.

This contradiction in the frog of White's results on *Necturus* made it seem necessary for us to study *Necturus* also. A series of twelve experiments has been completed, similar in all essential respects, in so far as we can judge, to those of White. The results are unequivocal and agree completely with those of the work upon frogs. The total molecular concentrations of glomerular urine and blood plasma were the same.

We have not the information upon which confidently to base an explanation of this discrepancy between our results and those of White: the one suggestion which we make concerns the possibilities of evaporation of glomerular fluid between its collection and the sealing of the Barger tube. It is to be hoped that future work will solve the difficulty.

Experiments with Barger's Method As Used by White for Comparing the Molecular Concentrations of Glomerular Urine and Plasma.

A clean, dry, glass capillary tube, 0.2 to 0.5 mm. inside diameter, was charged with the fluids to be examined: first, three successive droplets of plasma were drawn in by gentle suction, then the glomerular urine sample forced in from the collecting pipette, and finally three more droplets of plasma. The first and last drops of plasma were larger than the others and served as "barrier" drops. The tube was immediately sealed at both ends with plasticine, fastened to a microscope slide with rubber bands, and immersed

in water in a Petri dish which was kept at room temperature.[1] The lengths of the column of glomerular urine and the two adjacent columns of plasma on either side of this were measured accurately with a Zeiss ocular micrometer[2] within the next 2 hours. Similar measurements were made at the end of 24 and 48 hours. Increase or decrease in the column of glomerular urine by more than 1.5 per cent of its initial volume was regarded as a significant change indicating a real difference in molecular concentration between glomerular urine and plasma. Before applying the method to the actual problem of glomerular function, many control experiments were made in order to learn its accuracy and reliability. A summary of these follows.

1. When *all* of the drops in the capillary tube were taken from the same supply of fluid (serum, plasma, or salt solutions) the volume of the three central drops did not change significantly in 48 hours. With every set of tubes used in an actual experiment on glomerular urine, one capillary was included as a control in which all of the drops were taken from the same fluid—frog plasma. In consequence, we have records of 105 such controls, most of which were measured both at the end of 24 and of 48 hours. In 102 of these, changes in the central drop in 48 hours did not amount to more than 1.5 per cent of its initial volume; in only eight was the change more than 1.0 per cent; in the great majority the change was less than 0.5 per cent, a variation which is within permissible error of reading (2 scale divisions). It seems safe to conclude that alteration in the volume of the central drop of more than 1.5 per cent is significant.

2. 62 sets of measurements were made of tubes in which the central drop was composed of the same fluid (normal horse serum) as that of the adjacent drops, except for the addition of dry urea or sodium chloride in amounts to produce known change in molecular concentration. The results of a single representative set of such measurements are shown in Table I, in which are recorded the percentage changes in volume of the central (bold-faced

[1] White kept his tubes at a constant temperature of 34.5°. Barger (5), and Yamakami (6) who later made a thorough study of the method, worked at room temperature. Our control observations furnish evidence that no greater constancy of temperature than this is required.

[2] With the magnification used each scale division represented 2.39 μ.

figures) and the two immediately adjacent drops at the end of 24, 48, and 72 hours. In this instance NaCl was the substance added to increase the concentration of the fluids from which the central drops were taken.

When the concentration of the central drop differed by 10 per cent or more from that of those adjacent, an unmistakable change in its volume occurred within 24 hours. When the difference was only 5 per cent the change was unmistakable at the end of 48 hours. When the difference was only 2 per cent the change in volume, even after 72 hours, was inconclusive. The changes which occur in the volume of the central drop are roughly proportional to the differences in molecular concentration between it

TABLE I.

Changes in Volume Produced by Known Differences in Molecular Concentration.

NaCl added to central drop to increase molarity by:	Percentage change in volume of central and two adjacent drops after:								
	24 hrs.			48 hrs.			72 hrs.		
per cent									
0	0.0	−0.2	0.0	−0.2	−0.2	0.0	−0.2	0.0	0.0
2	+0.4	+0.9	0.0	+0.4	+1.3	−0.4	0.0	+1.5	−0.8
5	−0.6	+1.5	−0.6	−0.6	+2.4	0.0	−0.2	+2.4	0.0
10	0.0	+2.1	−1.3	−0.5	+3.0	−1.7	0.0	+3.4	−1.7
20	−0.6	+6.8	−1.7	0.0	+6.8	−1.4	+0.3	+7.9	−1.6

and its neighbors. It is on the basis of these volume changes that we obtain an approximately quantitative expression of the difference in molarity between glomerular urine and plasma.

3. In another series of controls, frog plasma was compared with watery solutions of sodium chloride of known strength. Three similar experiments were made with concordant results. One of them is summarized in Table II. A supply of heparinized frog plasma was obtained and a series of nine solutions of sodium chloride was made, increasing in concentration from 0.55 to 0.75 per cent. From each of these, a capillary tube was prepared in which the central drop was saline, the peripheral drops frog plasma. A tenth tube, containing only saline in the central and two adjacent drops, was included as a control. Readings were made over a period of 92 hours.

TABLE II.

Comparison of Frog Plasma with Different Concentrations of Salt Solution.

Concentration of NaCl solution.	Percentage change in volume of central (NaCl) and two adjacent drops (plasma) after:											
	18 hrs.			45 hrs.			72 hrs.			92 hrs.		
	Plasma.	NaCl	Plasma.	Plasma.	NaCl	Plasma.	Plasma.	NaCl	Plasma.	Plasma.	NaCl	Plasma.
per cent												
0.55	+2.1	**−1.4**	+1.1	+2.4	**−3.9**	+1.3	+3.8	**−5.0**	+2.1	+4.5	**−5.9**	+1.9
0.60	+1.4	**−1.8**	+1.0	+1.8	**−4.1**	+1.1	+2.2	**−4.3**	+1.1	+2.9	**−4.9**	+1.7
0.62	+0.6	**0.0**	+0.6	+0.8	**−1.2**	+0.9	+0.8	**−1.6**	+1.2	+0.8	**−1.9**	+1.4
0.64	+0.8	**−0.4**	0.0	+0.8	**−1.1**	−0.2	+1.4	**−1.6**	−0.2	+1.8	**−1.8**	+0.8
0.66	−0.5	**+0.2**	−0.2	−0.7	**−0.2**	−0.3	−0.7	**−0.2**	+0.2	−0.5	**0.0**	+0.5
0.68	−0.1	**+0.6**	−0.1	−0.4	**+1.2**	−0.6	−0.2	**+1.8**	−0.5	−0.2	**+1.9**	−0.7
0.70	−0.2	**+0.5**	−0.5	−0.5	**+0.6**	−1.3	−0.6	**+1.2**	−1.3	−0.6	**+1.4**	−1.6
0.72	−0.9	**+2.9**	−0.5	−0.6	**+3.6**	−0.6	−0.6	**+4.6**	−0.5	−0.5	**+5.1**	−0.3
0.75	−0.1	**+2.1**	−0.1	−0.5	**+3.3**	−1.7	−0.5	**+4.5**	−1.8	−0.3	**+4.9**	−1.5
Control, (all plasma).....	0.0	**+0.5**	−0.1	−0.2	**+0.4**	−0.4	−0.2	**+0.5**	−0.3	−0.1	**+0.5**	−0.4

In the experiment reported, it is evident that the molecular concentration of the plasma was approximately equivalent to that of a 0.66 per cent NaCl solution. The least degree of change in the central drop occurred in the tube containing this solution; in all those of lower strength, the central drop diminished in volume; in all of higher strength it increased. It will be noted that though the changes became progressively greater with time, no development occurred after 45 hours which altered conclusions reached at that time.

4. Ultrafiltrates of Plasma.—Ten experiments were made in which the molecular concentration of a protein-free ultrafiltrate was measured against that of the unfiltered plasma. In three, sterile horse serum was used; in seven heparinized frog plasma. In three instances the frog plasma was obtained from an animal at the end of an experiment in which glomerular urine had been collected. In these three, cellophane membranes were used; in all the others, parlodion sacs. The filtration pressure was 100 to 200 mm. of Hg. In every case the central drop in the capillary tube was ultrafiltrate. Readings were made after 48 hours.

The following results were obtained, expressed as percentage alteration in volume of the drop of ultrafiltrate after equilibration for 48 hours against the plasma: −1.7, −1.0, −0.3, −0.1, 0.0, +0.4, +0.7, +1.2, +1.6, +1.7; average = +0.25 per cent.

The conclusions drawn from the experience cited above are:

1. When all the drops with which the capillary tube is charged according to Barger's method are of fluid having the same composition, alterations in volume of the central drop in 48 hours do not amount to more than 1.5 per cent.

2. When the central drop of serum has the same composition as that of the other drops save for known additional urea or NaCl, a 5 per cent increase in molecular concentration can be reliably identified at the end of 48 hours. An increase of 2 per cent gives changes which are slightly greater than those which occur in control tubes but they are not great enough to be unmistakable.

3. The molecular concentration of frog plasma was found to be equivalent to that of a 0.66 per cent solution of NaCl in water. At the end of 48 hours it was unmistakably higher than that of a 0.60 per cent solution and unmistakably lower than that of a 0.72 per cent solution. Smaller differences than this are recognizable but not with so great certainty.

4. When an ultrafiltrate from serum or plasma is equilibrated against the unfiltered serum or plasma, change in volume in the drop of ultrafiltrate after 48 hours does not amount to more than 1.7 per cent.

These conclusions confirm White's belief that the method is adequate for the study of glomerular function. From our tests of the method we have adopted a figure of 1.5 per cent as an approximate expression of the maximum percentage change in the volume of the central drop of fluid which may be regarded as within experimental error.

Molecular Concentration of Glomerular Urine from Frogs. Details of Experiments.

In experiments on twenty-three healthy frogs (*Rana pipiens*) thirty-three collections of glomerular urine have been made and comparisons with plasma carried out. The experiments were made between February 11 and May 20, hence the list includes both winter and summer frogs. Weights of the frogs varied between 39 and 83 gm.; all except one were females.

The methods of preparing the frogs, identifying and blocking the tubule, collecting and transferring glomerular urine, and of collecting blood samples were identical with those described in the previous paper (4).

Each frog received 2 cc. of 0.6 per cent NaCl by subcutaneous injection before the dissection was begun. No intravenous injections were made, and no additional fluid given subcutaneously except in the interval between a first and second collection from the same frog.

Glomerular urine was collected against a positive pressure of from 2 to 11 mm. of Hg in twenty-eight experiments; in two the pressure was 0, and in three the mercury bulb was lowered 1 to 80 mm. below the level of the kidney in intentional efforts to contaminate the glomerular fluid with tubule contents. The fluid so collected varied in amount from 0.05 to 1.5 mg. The time required for the collection of glomerular urine varied from 10 to 75 minutes: twenty-five of the collections were complete in 40 minutes or less.

Proper position of the point of the pipette during collection was verified in every experiment by repeated observations of the be-

havior of the glomerular tuft when the leveling bulb was momentarily raised slightly.

Obstruction of the tubule to prevent possible contamination of glomerular fluid with tubule contents was made by pressure with a glass rod on the surface of the kidney over it. The position of the tubule was identified in twenty-six experiments by preliminary intracapsular injection of phenol red. In four, no preliminary injection was made, the rod being placed by guess, and the efficacy of the obstruction verified at the end of collection by intracapsular injection of dye. In four no blockage was attempted.[3] In eleven of the twenty-nine instances in which block was attempted the neck of the tubule was not clearly seen, hence the rod was placed over that portion of the proximal tubule which seemed to be nearest the glomerulus. These instances are noted in Table III as "tubule block not precise."

In twenty-one of the experiments the surface of the kidney was kept covered during collection with a 0.2 per cent solution of vital red in 0.7 per cent NaCl in order to enable detection of contamination of glomerular fluid with fluid which might have entered from outside the capsule.[4]

In experiments in which collection of the glomerular urine required only 30 minutes or less, only one sample of blood was taken, and this at about the middle of the collection. In longer experiments, two samples were taken, one shortly after the beginning, the other shortly before the end of collection. The blood samples were immediately sealed in the glass capillary tubes into which they were drawn from the cannula in the anterior abdominal vein, centrifuged, and set aside until the tube in which molecular concentrations were to be compared was made ready.[5]

[3] In one of these, the tubule was indentified but pressure upon it interfered with the glomerular circulation.

[4] In two of these instances a trace of vital red was detectable in the glomerular fluid, so faint as to suggest contamination amounting to about 1 per cent; it was then found, however, that vital red applied continuously to the surface of the kidney may be taken into the circulating blood and become detectable in the plasma; furthermore, after intravenous injection of vital red this dye makes its appearance in the glomerular fluid. Hence the two experiments alluded to were not discarded.

[5] Control experiments showed that Barger's method, as we have applied it, does not demonstrate difference in concentration between venous and

Particular pains were taken to avoid evaporation of the glomerular urine or of the plasma during the time which elapsed before the Barger tubes could be prepared. As soon as the collecting pipette was withdrawn from the capsule of Bowman, the stop-cock being closed, its tip was wiped first with moist, then with dry cotton. Then the tube containing plasma was opened over the surface of water in a bowl, one end capped loosely with a moist glass tube, and 3 droplets of plasma from the other end sucked into the Barger tube. That end was then capped, the Barger tube transferred to the stage of the microscope, and the glomerular urine delivered into it adjacent to the last droplet of plasma. Then, after the four droplets had been sucked a little further into the tube, 3 more droplets of plasma were successively taken in and the ends of the tube sealed. The whole performance was usually completed within 3 minutes. Nine control experiments showed that plasma left in a covered capillary tube for this length of time did not concentrate to a significant extent (*i.e.*, a droplet of this plasma did not increase in volume by more than 1.5 per cent when compared with plasma from a freshly opened capillary tube). Other control experiments, to test the possibility of evaporation of the glomerular fluid during its 5 minute stay in the pipette, showed no alteration in molecular concentration of saline solutions retained in the pipette for periods as long as 20 minutes.

In experiments in which more than one sample of blood was taken, the glomerular urine was compared directly with one specimen of plasma only. In a separate tube the two plasma specimens were compared. This procedure was followed in twelve experiments. In nine of these the two plasma specimens from a single experiment were identical in concentration. In only one was the result such as to indicate a difference in molecular concentration of the two of more than 10 per cent, and in this instance our precautions against evaporation were imperfect. We do not believe that significant alterations in the molecular concentration of circulating plasma occurred during the period of our glomerular collections. This belief is supported by control experiments

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arterial blood, simultaneously collected, and that no demonstrable effect on concentration is produced by the amounts of heparin we have used or by loss of CO_2 during the manipulations of the venous blood.

and by data on the electrical conductivity of plasma which appear in the following paper.

In six of the experiments 3 mg. of phenol red were injected with the salt solution subcutaneously before the dissection was begun; enough glomerular urine was collected to permit estimation both of molecular concentration and phenol red in comparison with plasma. In seven experiments enough glomerular urine was collected to allow estimation of both molecular concentration and electrical conductivity.

Results.

The results of thirty-three experiments on twenty-three frogs are assembled in Table III.[6] The average change in volume of the glomerular urines, after 48 hours' equilibration with plasma, is -0.40 per cent. If the result of Experiment 19 is omitted the value is $+0.35$ per cent. These changes are well within experimental error.

It seems not only proper, however, but obligatory to evaluate the results on the basis of technical perfection with which the experiments have been performed. In an experiment which includes so many manipulations, each of which must be carried out with precision if error is to be avoided, it is especially important to be rigidly critical and uncompromising in rejecting results which may have been contaminated by detectable inadequacies or uncertainties of technique.[7]

Therefore the results have been arranged in groups based on thorough search through the records of the experiments for indications of missteps. Group 1 contains twenty-one experiments in which we find no fault. In every one the condition of the animal

[6] The abbreviations "P. R., V. R." in the column of "Notes" in Table III mean that the tubule was identified by intracapsular injection of phenol red and that during glomerular urine collection the surface of the kidney was irrigated with a solution of vital red. Figures for volume of glomerular urine are only approximate. In some instances only a fraction of the amount collected was used in the Barger estimation.

[7] A group of eleven experiments made between January 18 and February 5 has been discarded because they contained various sources of inaccuracy which were discovered and avoided in the later experiments; ten out of the eleven seemed to show that the glomerular fluid was less concentrated than the plasma.

was satisfactory, dissection was made without mishap, puncture of capsule cleanly and accurately made, and collection of glomerular fluid uninterrupted; blood specimens were taken without untoward incident, transfer of plasma and glomerular fluid to the Barger tube made so quickly and under such circumstances as to compel belief that significant concentration by evaporation did not occur. In all but four the tubule was obstructed with certainty; in one of these (Experiment 10) the obstruction was omitted because it interfered with the circulation; in the other three (Experiments 23 to 25) it was intentionally omitted in a deliberate effort to contaminate the collected glomerular fluid with fluid from the tubule.

Circulation in the glomerular capillaries was exceedingly active with the exceptions noted in Table III; in no case was it so unsatisfactory as to indicate that the experient should be thrown out because of this.

In all except three of the twenty-one the change in volume of the glomerular fluid was within the limits of the values which control experiments described above have set as insignificant. In ten of these the direction of the change was +, in ten −; in one there was no change. The average of the twenty-one experiments is −1.1; if Experiment 19 be excluded the average is +0.1. In one experiment (No. 3) an increase in volume of 3.8 per cent occurred, in another (No. 19) a decrease of 24.6 per cent. It is to be noted that Experiment 3 was the third of a series of three on a single frog, of which the first two showed equality of glomerular fluid and plasma. It is difficult to believe that a "secretory power" of the glomerulus should become apparent only after 3 hours. No specific reason for the gross divergence of values found in Experiment 19 can be assigned. Electrical conductivity measurements on this same specimen also showed its hypotonicity. The difference is of the same order as that which is found when bladder urine is compared with blood plasma by the Barger method.[8] The result is analogous to the one cited in the previous paper (Experiment 11, p. 491) in which the phenol red concentration of supposedly glomerular fluid was found to be some 20 times as great as that of plasma. We think it probable that in this ex-

[8] In two experiments, bladder urine compared with plasma by Barger's method gave changes in volume of −18.7 and −20.5 per cent.

TABLE III.

Comparisons of Total Molecular Concentrations of Glomerular Urine and Blood Plasma from the Frog.

Experiment No.	Date.	Glomerular urine.			Barger tube measurements and change after 48 hrs.			Percentage change in volume of glomerular urine.	Notes.*
		Time.	Volume.	Pressure.	Plasma.	Glomerular urine.	Plasma.		
Group 1. Experiments with no known technical fault.									
	1929	*min.*	*c.mm.*	*mm.Hg*	*scale divisions*	*scale divisions*	*scale divisions*	*per cent*	
1	Feb. 11	27	0.4	+2	623 +7	559 +2	496 +4	+0.4	P. R., V. R. Tubule block precise.
3	" 11	26	0.18	+2	641 −3	422 +16	599 +1	+3.8	Same frog as in Experiments 1 and 2, different glomerulus. P. R., V. R. Tubule block not precise. Circulation slow during part of collection.
5	" 15	32	0.18	+3	291 +8	530 −8	523 −4	−1.5	P. R., V. R. Tubule block precise.
6	" 15	21	0.09	+3	600 −1	397 +1	317 +4	+0.3	Same frog as in Experiment 5, different glomerulus. P. R., V. R. Tubule block precise.
9	" 22	24	0.4	+3	433 +3	1016 +5	837 −1	+0.5	P. R., V. R. Tubule block precise.
10	" 22	39	0.27	+11– +5	541 +5	626 −4	764 +2	−0.6	Same frog as in Experiment 9, different glomerulus. P. R., V. R. Tubule not blocked because of interference with circulation.
11	" 25	13	0.3	+5	350 +2	803 −2	473 +1	−0.2	P. R., V. R. Tubule block precise.
14	" 27	17	0.5	+5	601 0	1021 +4	962 +2	−0.4	" " " " "

16	Feb. 28	31	0.18	+5	449 +5	540 −6	681 +4	−1.1	P. R., V. R. Tubule block precise. Circulation somewhat slow.
17	Mar. 5	18	0.2	+3	305 +7	834 −8	424 −1	−0.9	P. R., V. R. Tubule block not precise. Circulation slow.
19	" 11	47	0.8	+3	513 +19	353 −87	760 +49	−24.6	Same frog as in Experiment 18, different glomerulus. Electrical conductivity also. P. R., V. R. Tubule block not precise.
20	" 12	46	1.0	+3	497 +5	965 +6	530 −11	+0.6	Electrical conductivity and phenol red comparison also made. P. R. Tubule block not precise.
21	" 13	60	0.7	+3	477 +4	610 +6	511 +3	+1.0	Electrical conductivity also. P. R., V. R. Tubule block precise.
23	" 15	20	0.08	−40	532 +9	306 0	734 −2	0.0	Tubule not blocked.
24	" 19	30	0.3	−80	605 +8	487 −1	478 −2	−0.2	" " " Circulation slow. Second collection from this frog.
25	" 20	20	0.65	−1	484 +6	421 −4	420 0	−0.9	Electrical conductivity also. Third collection from this frog. Tubule not blocked. Ureter ligated.
29	Apr. 4	22	0.2	+3	469 +11	674 −11	411 +3	−1.6	Phenol red comparison also made. Tubule block not precise.
30	" 4	35	0.4	+3	497 +2	766 −4	523 +3	−0.5	Same frog as in Experiment 29, different glomerulus. Phenol red comparison also made. Tubule block not precise.
31	" 5	20	0.5	+3	590 +3	956 +4	425 −5	+0.4	Phenol red comparison also made. Tubule block precise.

* The abbreviations "P. R., V. R." mean that the tubule was identified by intracapsular injection of phenol red and that during glomerular urine collection the surface of the kidney was irrigated with a solution of vital red.

TABLE III—*Concluded.*

Experiment No.	Date.	Glomerular urine.			Barger tube measurements and change after 48 hrs.			Percentage change in volume of glomerular urine.	Notes.*
		Time.	Volume.	Pressure.	Plasma.	Glomerular urine.	Plasma.		
Group 1. Experiments with no known technical fault—*Concluded.*									
	1929	*min.*	*c.mm.*	*mm.Hg*	*scale divisions*	*scale divisions*	*scale divisions*	*per cent*	
32	Apr. 5	35	1.0	+3	573 0	854 +8	598 −2	+0.9	Same frog as in Experiment 31, same glomerulus. Phenol red comparison also made. Tubule block precise. Circulation somewhat slow.
33	May 20	75	1.4	+5	490 −1	641 +8	521 +5	+1.2	Electrical conductivity also. P. R. Tubule block precise.
Average excluding experiment 19								+0.1	
Group 2. Experiments in which mixed glomerular fluid and plasma were compared with plasma.									
2	Feb. 11	40	0.2	+2	683 +11	662 −1	478 +3	−0.1	Same frog as in Experiment 1, different glomerulus. P. R., V. R. Tubule block precise.
7	" 16	60	0.14	+3 0	606 +6	747 −4	498 −6	−0.5	P. R., V. R. Tubule block precise.
12	" 25	46	0.12	+5	1034	531 +6	665 −4	+1.1	Same frog as in Experiment 11, different glomerulus. P. R., V. R. Tubule block not precise.
22	Mar. 14	72	0.6	+4	603 +4	760 −4	631 −3	−0.5	Second collection from this frog. Electrical conductivity also. P. R., V. R. Tubule block not precise.
Average								0.0	

Group 3. Experiments in which volume of glomerular fluid tested was very small.									
8	Feb. 20	10	0.05	0	787 +6	199 +6	636 +1	+3.0	P. R., V. R. Tubule block precise. Reading at 22 hrs.
18	Mar. 11	38	0.6	+3	789 +2	141 +6	534 −2	+4.3	Electrical conductivity also. P. R., V. R. Tubule block precise.
27	Apr. 2	30	0.3	+3	456 −3	195 +7	406 +9	+3.6	Same frog as in Experiment 26, different glomerulus. Phenol red comparison also made. P. R. Tubule block precise.
Average								+3.6	
Group 4. Experiments containing various possible sources of error.									
4	Feb. 13	22	0.15	+2	328 +3	228 +14	348 −6	+6.1	Second collection from same glomerulus, capsular tear possible. Short drop of glomerular fluid used. P. R., V. R. Tubule block precise.
13	" 26	36	0.13	+10	470 −3	368 +2	776 0	+0.5	Capsule probably torn. Delay in transfer of glomerular fluid. P. R., V. R. Tubule block not precise.
15	" 27	12	0.5	+5	723 +18	833 −35	518 +19	−4.2	Posterior wall of capsule and tubule beneath it punctured. P. R., V. R. Tubule block not precise.
26	Apr. 2	22	0.12	+3	606 +2	389 −4	842 +8	−1.0	Reading inaccurate, air with glomerular fluid in Barger tube. P. R. Tubule block precise.
28	" 3	46	0.29	+3	753 +13	496 −15	572 +6	−3.0	Posterior wall of capsule and tubule beneath it punctured. P. R. Tubule block not precise.
Average								−0.3	

periment as in that, the tip of the pipette entered a distal tubule lying underneath the punctured capsule and that collection was actually made from the tubule.

We regard the outcome of these twenty-one most perfect experiments as demonstrating essential identity of molecular concentration of glomerular fluid and plasma.

In Group 2 of Table III are four experiments in which the tip of the collecting pipette was unusually fine and in consequence the glomerular fluid flowed slowly down the wall of the Barger tube and mixed with the last droplet of plasma, previously introduced. In each case the determination of change in volume of the combined droplet of glomerular fluid and plasma against plasma alone was carried through. All other details of these four experiments were properly executed. We assume that while the magnitude of the change in volume was lessened its direction could not be; and since the results are wholly in accord with those of the first group of twenty-one they may very properly be regarded as confirmatory of them.

In Group 3 are collected the data of three experiments in which the molecular concentration of the glomerular fluid was apparently about 10 per cent greater than that of the plasma. In each of these the volume of glomerular fluid used in the Barger estimation was excessively small as the figures for length of column in the Barger tube show. For reasons set forth below we attribute these high concentrations to evaporation of the glomerular urine during and immediately after transfer from the collecting pipette: relative extent of surface becomes very great as volume of fluid decreases and error from this source is correspondingly magnified.

Finally there is a group of five experiments (Group 4) in which miscellaneous causes for suspicion of the results are to be found. It will be observed that the volume changes of glomerular urine in these is not consistent in either direction and that the average of the five indicates identity. They are not given the weight either for or against our conclusion that Groups 1 and 2 receive; but they present no obstacle to that conclusion.

Taking account of these criticisms, the conclusion which we draw is that in the frog essential equality exists between the total molecular concentration of glomerular fluid and that of plasma from which it is derived: the more perfect the experiment technically the more certain is this result to be obtained.

This conclusion is supported by the results of six experiments in which, phenol red having been injected, the concentration of this substance in the glomerular urine was determined in addition to the molecular concentration. The results of these experiments are collected in Table IV.

It will be noted that in every instance the glomerular concentration of phenol red is of the same order as that found in an ultrafiltrate from the plasma. With one exception the molecular concentrations of glomerular urine and plasma are the same and the exceptional experiment is taken from Group 3 of Table III in which the volume of glomerular urine examined was unusually small.

TABLE IV.

Comparisons of Total Molecular and Phenol Red Concentrations in the Same Collections of Glomerular Urine and Plasma from Frogs.

Experiment No.	Date.	Molecular concentration (change in volume of glomerular urine in Barger tube with plasma).	Phenol red in glomerular urine as percentage of that in plasma.
	1929	*per cent*	
20	Mar. 12	+0.6	67
29	Apr. 4	−1.6	75
30	" 4	−0.5	70
31	" 5	+0.4	71
32	" 5	+0.9	76
27	" 2	+3.6*	67

* This estimation is one of those criticized in Group 3 of Table III. The corresponding phenol red estimation was made on a larger fraction of the glomerular collection.

Further support is afforded by seven experiments of the series in which electrical conductivity of glomerular urine and plasma was determined. More specific allusion will be made to them in the following paper.

Molecular Concentration of Glomerular Urine from Necturus. Details of Experiments.

Freshly caught *Necturi* were kept in a tank of aerated water. They measured from 7 to 12½ inches in length and appeared normally active. Before dissection the brain was crushed by a

hemostat without trauma to the gills; the body was pinned upon the frog board and covered with damp cotton; head and gills were allowed to hang over the end of the board into a basin of water through which oxygen was kept bubbling; the body cavity was opened, the anterior abdominal vein reflected and a cannula inserted, the left kidney exposed, and observation made by reflected light. A preliminary intravenous injection of less than 1.0 cc. of 0.65 per cent NaCl was found necessary to insure an active glomerular circulation. In six animals the preliminary injection contained urethane to prevent movements during collection.

Twelve successful experiments were performed. The methods of collection and of measurement were like those described for the frog except that no attempt was made to block the tubule. Glomerular fluid was collected in all but one experiment against positive pressures of from 1 to 10 mm. of Hg and over periods of from 4 to 70 minutes. The collected fluid varied from 0.12 to 1.5 c.mm. in amount, and in four experiments was sufficient to permit determinations both of molecular concentration and electrical conductivity on the single sample. A single specimen of plasma was obtained in each experiment by incision of the mesenteric vein or posterior vena cava at the conclusion of the glomerular collection.

Three ultrafiltrates of *Necturus* plasma gave the following percentage changes in volume when equilibrated against the plasma: -1.7, -0.6, $+1.0$; average $= -0.4$.

Results.

Table V summarizes the results. In one experiment (No. 8) the glomerular fluid was markedly hypotonic to the plasma and conductivity measurements confirmed this finding; but the mercury bulb of the collecting pipette was 2 mm. below the level of the kidney and during collection it was frequently lowered to test the correctness of the position of the tip of the pipette. The conditions of this experiment were therefore favorable to the entrance of tubular fluid into the capsule. In eight of the other eleven experiments the change in glomerular fluid volume after equilibration with the plasma was insignificant. In the remaining three, the changes indicate differences in molecular concentration of between 5 and 10 per cent; in these it is noticeable (as in Group 3 of the experiments with frogs) that the column of glomer-

ular fluid was unusually short in the two of those three which show an apparent hypertonicity to plasma; so that again we think that the result may have been influenced by evaporation. The average change in glomerular fluid volume in these eleven experiments was only +0.28 per cent.

These results indicate that in *Necturus*, as in the frog, the molecular concentration of glomerular urine is identical with that of plasma. They agree with our results on the glomerular elimination of dyes in frogs (4) and with our measurements of electrical conductivity both in frogs and in *Necturi* (7). They stand in direct contradiction to the results published by White.

In discussing Table III, we called attention to three experiments (Group 3) in which the volume of glomerular fluid transferred to the Barger tube was unusually small and in which hypertonicity was apparently demonstrated. The two exceptional results with *Necturus* show a similar coincidence. Control experiments have been made which show that when the volume of fluid is small considerable concentration can result from evaporation during the time required for its transfer and for introduction of the next droplet of plasma. Typical examples are as follows:

A. Two Barger tubes were charged with successive droplets of 0.65 per cent NaCl, all from the same supply, the central drop being delivered from a capillary pipette with a tip about 5μ inside diameter. Change in volume of the central drop was measured after 44 hours equilibration. In the first tube the initial length of the central drop = 152 scale divisions; increase in 44 hours = 7 scale divisions = 4.6 per cent. In the second tube the initial length of the central drop = 773 scale divisions; increase = 4 scale divisions = 0.5 per cent.

B. A droplet of distilled water was delivered from the capillary pipette into the end of a capillary tube, 0.4 mm. in diameter, so that its outer meniscus remained about 1 mm. from the end of the tube. The length of the drop decreased by 8 scale divisions during the 1st minute and at the average rate of 8.4 scale divisions for 5 minutes.

In a similar experiment, the drop was placed at the extreme end of the tube, but during evaporation it receded. The length of the drop decreased by 32 scale divisions in the 1st minute; thereafter at a decreasing rate to 14 scale divisions in the 5th minute.

TABLE V.

Comparisons of Total Molecular Concentrations of Glomerular Urine and Blood Plasma from Necturus.

Experiment No.	Date.	Glomerular urine.			Barger tube measurements and change after 48 hrs.			Percentage change in volume of glomerular urine.	Notes.
		Time.	Volume.	Pressure.	Plasma.	Glomerular urine.	Plasma.		
	1929	*min.*	*c.mm.*	*mm.Hg*	*scale divisions*	*scale divisions*	*scale divisions*		
1	Apr. 18	7	0.3	+6	862 +7	476 0	323 +4	0.0	
2	" 19	22	0.12	+10	418 +2	203 +1	516 +1	+0.2	Circulation slow.
3	" 20	10	0.3	+10	495 +2	636 −10	524 +9	−1.6	Capsule torn.
4	" 22	10	0.2	+6	587 +5	533 0	723 −4	0.0	
5	" 26	10	0.2	+4	424 +3	358 −2	674 +1	−0.6	
6	June 4	25	0.14	+9	716 −1	306 +4	559 +5	+1.3	Capsule probably torn.
7	" 5	38	1.0	−2	710 +4	680 −4	708 +7	+0.6	1 cc. 25 per cent urethane, intravenously. Electrical conductivity comparison also.
8	" 5	27	0.8	−2	719 +18	308 −65	721 +46	−21.1	Same *Necturus* as in Experiment 7, different glomerulus. Hg bulb frequently lowered during collection. Electrical conductivity also.

9	June 6	45	0.8	+3	485 0	171 +4	463 −5	+2.4	Second collection, different glomerulus. 1 cc. 12.5 per cent urethane, intravenously. Electrical conductivity also. Reading at 19 hrs.
10	" 10	70	0.9	+4	950 0	463 +5	703 −1	+1.1	Second collection, different glomerulus. 1.5 cc. 12.5 per cent urethane intravenously.
11	" 14	27	1.5	+1	454 +4	229 +4	635 +5	+1.8	Second collection, different glomerulus. 1.0 cc. 12.5 per cent urethane intravenously. Electrical conductivity also. Reading at 26 hrs.
12	" 15	4	0.27	+3	694 +9	607 0	727 +1	0.0	Third collection, different glomerulus. Capsule torn. 1.5 cc. 12.5 per cent urethane.
Average (excluding Experiment 8)......................								+0.47	

In another experiment the outer meniscus of the drop remained at the end of the tube: in the first 30 seconds its length decreased by 41 scale divisions (6.7 per cent), in the 1st minute by 78 scale divisions (12.8 per cent), and after 9 minutes it measured only one-fifth of its original volume.

C. The time required for 0.7 c.mm. of water to flow from the collecting pipette into the capillary tube under a pressure of 60 cm. of Hg was 61 seconds when the inside diameter of the tip of the pipette was 4.4μ; 6 seconds when it was 10μ.

From 25 to 30 seconds were required to remove the capillary tube from the stage of the microscope after delivery of the glomerular urine into it and to take in the next succeeding droplet of plasma.

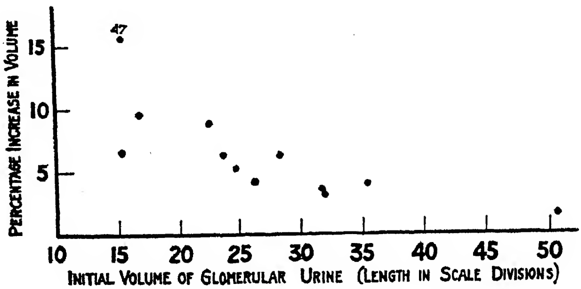

Chart 1. Data taken from White's Table 2 ((1) p. 203). With the one exception designated, times of equilibration of glomerular urine against plasma were from 18 to 25 hours.

These control experiments show that under certain circumstances the danger of concentration of the glomerular urine may be very great during the brief time which must elapse before the next drop of plasma can be introduced into the Barger tube. They were suggested by the coincidence of high concentration of glomerular urine and small volume used in the estimation in the three experiments of Group 3 of Table III; they give reasonable assurance, not only that evaporation was responsible for those results and for the two highest concentrations found in *Necturus*, but also that it did not constitute a source of serious danger when the volumes of glomerular fluid were considerably larger.

The question naturally arises whether this source of error can

have been influential in White's experiments. He states that it is inconceivable that significant evaporation could take place during the few seconds interval which we have been discussing; and six control experiments made in another connection bear out this statement. His volumes of glomerular urine were not as small as those of which we are speaking. Yet the diameter of his Barger's tubes was approximately twice that of ours; hence the exposed surface at or near the end was some 4 times as great as in ours. Not only is this true after the fluid has been introduced, but also during its passage from the pipette into the Barger tube, before the lumen is closed by the meniscus, the relation of exposed surface area to volume is far greater than in a smaller tube. Furthermore, when White's results are plotted as in Chart 1 a distinct impression is given that the degree by which the molecular concentration of glomerular urine appears to exceed that of plasma is inversely related to the volume of glomerular urine examined.

SUMMARY.

A study has been made of Barger's microscopic method of estimating molecular weights as used by White in comparing the total molecular concentrations of glomerular urine and blood plasma from *Necturus*. White's belief in its usefulness for this purpose was confirmed.

In twenty-five comparisons of glomerular urine and blood plasma from frogs, the total molecular concentrations of the two fluids were found to be the same within the limits of permissible experimental error. In eight experiments in which differences in concentration were greater than could be ascribed to permissible error, the differences were not consistently in one direction and in all but one could be attributed to identified flaws in experimental technique.

In six experiments, phenol red was injected and its concentration in the two fluids also determined; in seven others electrical conductivity was also measured. The results of these additional estimations agreed well in showing that glomerular urine has the composition of a protein-free filtrate from plasma.

Twelve similar comparisons were made of the total molecular concentrations of glomerular urine and blood plasma from *Necturus maculosus*. The results were like those on the frog. In four

instances they were supported by the concordant results of electrical conductivity measurements.

The outcome of these experiments conflicts with that of twelve experiments made by White on *Necturus* which seemed to show that the glomerular urine is more concentrated than the plasma from which it is derived. Our experience suggests that it is easy to underestimate the importance of dangers of evaporation.

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THE ELECTRICAL CONDUCTIVITY OF GLOMERULAR URINE FROM THE FROG AND FROM NECTURUS.*

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If protein-free fluid is eliminated from the blood through the glomerular membrane by a process of simple filtration, its electrical conductivity should differ from that of the plasma only by an amount representing the retarding effect of the protein on the ions. The work of Bugarszky and Tangl (1) and Gram and Cullen (2) has shown that the degree of this retardation is from 2.2 to 2.5 per cent per 1 per cent of protein. The protein content of frog plasma is of the order of 3 per cent: hence, on the assumption of glomerular filtration, we might expect to find that the conductivity of the glomerular urine of frogs is about 7 per cent greater than that of the plasma from which it is derived.

The protein effect can be measured quantitatively by ultrafiltration. It is scarcely practicable, during glomerular urine collections, to take samples of blood large enough to yield sufficient plasma for ultrafiltration. Hence our ultrafiltration experiments have been made, some on blood plasma obtained from animals after the completion of a glomerular urine collection, others on plasma from animals used only for bleeding. No systematic difference was apparent between the results of the two groups and accordingly they are treated as one series, with which the series on glomerular urines is compared.

The outcome of the experiments has yielded the conclusion that the difference in conductivity between blood plasma and

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† Traveling Fellow of the Rockefeller Foundation, 1928–29.

glomerular urine is the same as that between blood plasma and an ultrafiltrate from it.[1]

Methods.

Preparation of Animals.—This was performed as described in the previous papers (3,4). With a few exceptions, noted in the tables, the tubule was blocked during collection of glomerular urine from frogs. No attempt was made to block the tubule in *Necturus*. In the great majority of experiments the glomerular urine was collected against a positive pressure in the capillary pipette maintained by the Hg leveling bulb. In all but three experiments destruction of the brain of *Necturus* by crushing was insufficient to suppress muscular movements of the animal: small amounts of urethane[2] in these instances were injected intravenously to secure complete immobilization.

Measurement of Electrical Conductivity.—The circuit used was that of the standard Kohlrausch bridge. It was fed from a 1000 cycle audiofrequency generator made by the General Radio Company, and the null point was determined by head phones of the usual pattern. Owing to the special circumstances attending the use of our conductivity cell, such as the small size of the electrodes and the necessity of drying them, the null point was inclined to be flat until a $0.01\mu F$ condenser was connected across the resistance box used as standard resistance, when it became as sharp as could be desired. The impedance of this combination was, of course, slightly less than that of the box alone, but the difference was too small to be of consequence, particularly as all our measurements were essentially measurements of small differences.

Our standard practice was to adjust the resistance box until the minimum sound intensity was obtained with the slide wire at mid-point, or until a sharp increase in intensity occurred symmetrically on each side of it. An alteration of the setting of the box by as little as 0.5 per cent could be detected without difficulty.

The cell was constructed as follows (Fig. 1): Two blocks of

[1] To avoid cumbersome terminology, we propose to call these differences the ultrafiltrate protein effect and the glomerular urine protein effect respectively.

[2] From 0.125 to 0.250 gm. was injected early in the dissection.

vulcanite (A and B) were shaped as shown in the figure and small holes drilled through them near one edge. In one of the blocks (A) the hole was enlarged at one end so as to form a recess (C) in the face of the block 0.5 mm. deep, 0.75 mm. wide, and 1 mm. long,

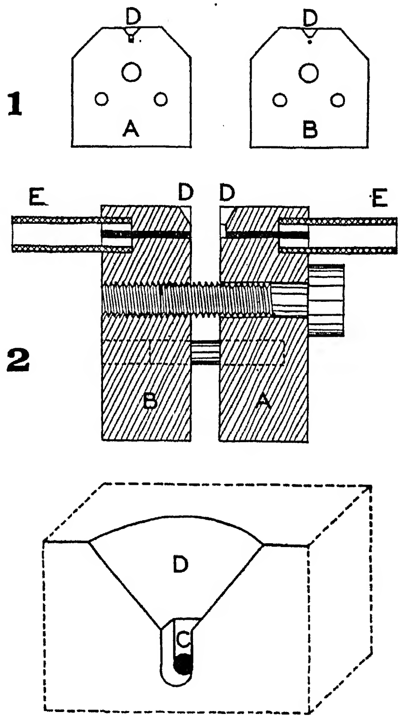

FIG. 1. Conductivity cell. Section I shows the faces of the two blocks which make the cell (actual size), Section 2 shows the construction, and below is an enlarged diagram of the recess which holds the fluid. A and B are vulcanite blocks which form the cell. C is a recess in the face of one of the blocks, A, which forms a cavity when A and B are fitted together. D is a conical opening to the cavity. E, E are glass tubes filled with mercury to make connection between the platinum wires and the remainder of the circuit.

extending from the hole through the block towards the edge. In each of these holes a piece of platinum wire, as long as the thickness of the block, and about 0.3 mm. in diameter was sealed with sealing-wax, care being taken that the end of the wire was flush

with the bottom of the recess in one case and with the face of the block in the other. The other end of each hole was enlarged to take a short glass tube (*E*, *E*) also sealed in with sealing-wax, which served to protect the outer end of the platinum wires and, when filled with mercury, to make connection with the remainder of the circuit.

The two blocks, faced so as to fit perfectly against one another, were located by two pins and held firmly together by a thumb-screw, so that a small cavity, having a platinum electrode in each face, was formed in what was virtually one vulcanite block. A conical hole (*D*, *D*) was then made in the face of the block nearest to the cavity, so that it could be reached from the outside.

In practice, the final shaping of the recess was done with sealing wax, as the original cavity was too large. This was not altogether satisfactory, as the surface gradually disintegrated from being continually wet, and formed cavities which caught air bubbles when the cell was being filled, so that eventually the whole of the wax had to be taken out and reinserted.

Each electrode was coated with platinum black by placing a small drop of platinum chloride solution (about 2 per cent) on it and dipping the end of an auxillary wire into the drop. Good results were obtained when 3 volts were used and the current reversed every 10 seconds for 2 or 3 minutes.

It is essential, of course, that the cell be perfectly dry before being filled since there was never enough glomerular urine to allow rinsing with this; but since the drying of a blacked electrode alters its surface resistance, we seemed to be faced with a dilemma. At first we contented ourselves with repeated determinations of the cell constant by means of 0.1 M KCl, and assumed that the continued rise in its value was uniform with time. Later, however, we found that by keeping the cell in distilled water and only drying it immediately before filling, we could reduce the drift and the cell constant remained steady, within experimental error, during the whole of a day's series of measurements. It showed a steady rise, with gradual flattening of the end-point, however, so that the electrodes had to be reblacked every week or so.

The cell was filled under a binocular dissecting microscope through the conical opening mentioned, by means of a fine pipette.

A piece of small bore glass tubing was drawn out for about 100 mm. to a diameter of about 0.2 mm. or a little less. It was cut at points about 20 to 30 mm. from the beginning of the constriction, where it began to be sensibly uniform in diameter, and the central portion discarded. The ends of the two pipettes so formed were bent at right angles about 5 mm. from the small end. It is essential that the ends be cleanly and squarely cut. Each pipette was furnished with a short piece of rubber tubing, so that it could be attached to a tap, held conveniently close to the microscope stage. This tap communicated at its other end with a reservoir of mercury about 80 mm. above the level of the stage, a fine capillary in the connecting tube preventing rapid movements and making the system more controllable. A side tube which could be clamped when necessary allowed the pipette to be attached or removed without discharging its contents.

These pipettes (of which it was advisable to have several available as they were easily broken) were filled from the micro pipette used for collecting the glomerular urine in the same way as were the capillary tubes used in the previous work. Evaporation was prevented during the time taken to carry them to the conductivity apparatus by having a piece of damp cotton in the larger portion, and by supporting them in a small test-tube immediately above the surface of water, as soon as they were removed from the microscope stage. A control experiment showed that there was no appreciable loss by evaporation in 10 minutes.

In filling the cell, the pipette was attached to the tap and the side tube clamped. The pipette was then carefully inserted into the cell, under the microscope, until its tip touched the bottom, and the tap turned on. It was found advisable to have the mercury reservoir initially too low to drive the fluid out of the pipette, and gradually to raise it. As soon as fluid was seen to leave the tip, the pipette was gently withdrawn at such a rate that when the last portion of its contents was blown out, the air following it did not bubble into the portions already discharged. Difficulty was sometimes experienced with small air bubbles which clung to the walls of the cell and the electrodes, but these could usually be removed by withdrawing the fluid into the pipette again and repeating the filling more slowly.

In most cases, two samples of blood were taken in capillary

tubes during the collection of each sample of urine, as already described (3). Plasma from these was drawn into a transfer pipette and placed in the cell in exactly the same way as was the glomerular urine. The average difference between the conductivities of two such samples was 3.5 per cent; the two greatest differences were 8 per cent and 8.5 per cent, but in both of these we had reason for suspecting that the plasma sample with the higher conductivity had had an opportunity for evaporation before being inserted into the cell: more weight was accordingly put on the other, when calculating the mean.

All our results, except where otherwise stated, are expressed in terms of specific conductivity $\times 10^4$ reduced to 20°. No special control of temperature was made, but the room temperature very rarely changed by as much as 1° between the determination of the conductivity of the plasma and that of the glomerular urine. Hence a small error in the value of the temperature coefficient used in the reduction of the values to 20° (that of 0.1 M KCl) would only affect the difference between them to an insignificant degree. Repeated determinations of the cell constant at various temperatures controlled any possible errors arising from changes in the dimensions of the cell.

Ultrafiltrations were performed in a simple but effective apparatus. A piece of cellophane was tied tightly over the end of a short length of glass tube about 1 cm. in diameter, a piece of rubber tube slipped over the glass assisting in making a tight seal. This was held just clear of the surface of water in a large test-tube. The plasma[3] was poured into the inner tube onto the membrane and covered with liquid paraffin to prevent evaporation. A pressure of about 200 mm. of Hg was then applied. The advantage of using cellophane over collodion is that it can be dried between filtrations, so that only a minimum of the ultrafiltrate need be discarded. It was our practice to continue ultrafiltration until the several droplets that first formed on the membrane had run together, to remove this with a piece of clean filter paper, and to continue for another 10 minutes, when enough had come through for several determinations of conductivity, if necessary. A sample of the plasma was obtained by pouring out the contents of the

[3] The method is applicable to amounts of plasma as small as 0.25 cc.

ultrafilter into a small dish and picking it up in a capillary tube; a layer of liquid paraffin covered it continually and prevented evaporation. The adequacy of cellophane as a semipermeable membrane for our purpose was checked by comparison against collodion. Complete agreement was obtained.

Controls.

1. Four solutions of NaCl were made up and titrated against $AgNO_3$ in the usual way. The electrical conductivity of these was then determined on various occasions during the course of the work in exactly the same way as was that of the glomerular urine, and often just before a glomerular sample was ready. The results, converted into terms of per cent of NaCl, are given in the following tabulation.

Solution No.	1	2	3	4
Concentration by titration	0.585	0.635	0.670	0.695
" " conductivity	0.591	0.631	0.670	0.700
Difference	+1.03	−0.63	0.00	+0.72

In all, seven measurements were made on Solution 1, eight on Solution 2, three on Solution 3, and four on Solution 4, those on Solutions 1 and 2 yielding a standard deviation of 0.006 or 1 per cent. The apparent small systematic error, which is not to be regarded as significant, probably arises from slight errors in the titration and the preparation of the standard solutions.

2. As a check on the whole technique, a frog kidney was perfused with saline solution and a glomerular collection made. This had a conductivity of 113, corresponding to 0.69 per cent NaCl, while the perfusion fluid had a conductivity of 110, corresponding to 0.67 per cent NaCl. This latter was made up to be 0.65 per cent NaCl (not very accurately) and a small quantity of $NaHCO_3$ added, so the absolute value of the conductivity measurement may be considered satisfactory.

3. Further evidence of the reliability of the method is obtained from a consideration of the results of the experiments in which the same sample of glomerular urine was analyzed both by the Barger method, as described by Walker, and by estimation of elec-

trical conductivity. These five experiments, given in Table VI show good agreement.

4. As as control on the ultrafiltration technique, a saline solution with a conductivity of 100 was put in the ultrafilter and the solution coming through was found to have a conductivity of 99.5.

Results.

Ultrafiltrates from Frog Plasma.—The conductivity of frog plasma and an ultrafiltrate from it has been measured in ten experiments. Four of the frogs were used only for supplying blood; from six the blood was taken after the completion of a collection of glomerular urine. The results are given in Table I.

TABLE I.

Electrical Conductivity of Ultrafiltrates from Frog Plasma.

Date.	Conductivity.			NaCl equivalents.			
	Plasma.	Ultrafiltrate.	Difference.	Plasma.	Ultrafiltrate.	Difference.	
1929	$K_{20°} \times 10^4$	$K_{20°} \times 10^4$	*per cent*	*gm. per 100 cc.*	*gm. per 100 cc.*	*gm. per 100 cc.*	
Feb. 5	92	99.5	+8.2	0.56	0.61	0.05	For blood only.
" 6	95.5	101.5	+6.3	0.58	0.62	0.04	" " "
" 14	105	110	+4.8	0.64	0.67	0.03	After glomerular urine collection.
" 10	98	110	+12.2	0.60	0.68	0.08	" "
" 18	98	108	+10.2	0.60	0.66	0.06	For blood only.
Mar. 4	102	109	+6.9	0.62	0.66	0.04	After glomerular urine collection.
" 13	97.5	106	+8.7	0.60	0.65	0.05	" "
" 14	102	108.5	+6.4	0.62	0.66	0.04	" "
" 22	102	108	+5.9	0.62	0.66	0.04	For blood only.
May 27	90	98	+8.9	0.55	0.60	0.05	After glomerular urine collection.
Average.			+7.9			0.05	

The results show that the conductivity of an ultrafiltrate is greater than that of the plasma from which it is derived by from 4.8 to 12.2 per cent; the average of ten determinations is 7.9 per cent. Expressed in terms of NaCl, the conductivity of an ultrafiltrate exceeds that of its plasma by the equivalent of 0.03 to 0.08 per cent of NaCl.

The figures for conductivity of plasma from frogs used only for bleeding may be taken to represent blood at the beginning of a glomerular urine collection; the other plasma specimens were from blood taken at the end of a collection: no systematic difference between them can be detected. That the conductivity may, however, change considerably with changes in protein content consequent upon hemorrhage and absorption of salt solution, is shown by the following experiment (Table II) in which a frog was repeatedly bled out and allowed to regain fluid by absorption from the lymph spaces.

Glomerular Urine from Frogs.—Twenty-six experiments on 18 frogs have been made. The measurements of electrical conductivity collected in Table III have been divided into two groups: thirteen experiments in which we are not aware of the occurrence of any technical errors either in the collection of the urine or in the determination of the conductivity (Group A), and thirteen into which slight errors, of known direction and sometimes of approximately known magnitude, have crept (Group B). Most of those in Group B were placed under suspicion either because there was some doubt whether the cell was quite full enough to give a true reading, because an air bubble was trapped in the cell when it was being filled, or because the glomerular urine was so long exposed to the air during transfer that some evaporation might have taken place.

TABLE II.

Effect of Blood Dilution on Conductivity of Plasma.

Blood sample No.	Time.	Conductivities.			Notes.
		Plasma.	Ultra-filtrate.	Difference.	
	min.	$K_{20°} \times 10^4$	$K_{20°} \times 10^4$	*per cent*	
1	0	95.5	101.5	6.3	Bled 1.3 cc.
2	10	96.5	100.5	4.1	" 1.3 " 2 cc. saline subcutaneously.
3	20	97.0	101.0	4.1	Bled 1.2 cc. 2 cc. saline subcutaneously.
4	30	98.5	101.0	2.5	Bled 1.1 cc.

The results of Group A are more consistent and may be expected

TABLE III.

Electrical Conductivity of Blood Plasma and Glomerular Urine from Frogs.

Experiment No.	Date.	Glomerular urine collection. Time.	Glomerular urine collection. Pressure.	Blood. $\frac{\text{Time*}}{\text{R. B. C. volume}}$.	Conductivities. Plasma.	Conductivities. Glomerular urine.	Conductivities. Difference.	NaCl equivalents. Plasma.	NaCl equivalents. Glomerular urine.	NaCl equivalents. Difference.	Notes.
Group A. Experiments with no known technical faults.											
	1929	*min.*	*mm. Hg*	$\frac{\textit{min.}}{\textit{per cent}}$	$K_{20^\circ} \times 10^4$	$K_{20^\circ} \times 10^4$	*per cent*	*gm. per 100 cc.*	*gm. per 100 cc.*	*gm. per 100 cc.*	
7	Mar. 4	60	+3	10, 90 **37.5**	95	105	+10.5	0.58	0.64	0.06	
10	" 6	115	+3	15, 87 **32, 32**	99	109	+10.1	0.60	0.67	0.07	
18	" 13	60	+3	15, 70 **40, 27**	89.5	99.5	+11.2	0.54	0.60	0.06	
19	" 14	92	+4	20, 80 **50, 54**	98.5	107	+8.6	0.60	0.66	0.06	
22	" 19	38	−80	18 **42**	99	104.5	+5.6	0.60	0.64	0.04	Tubule open.
23	" 20	57	−1	19, 49 **34, 36**	95	104	+9.5	0.58	0.64	0.06	" "
24	" 20	57	−1	35 **33**	105	113.5	+8.1	0.64	0.69	0.05	" " Same frog as in Experiment 23.

* "Time" of blood samples is expressed as minutes after beginning glomerular urine collection. Figures in bold-faced type are the hematocrit readings.

28	May 21	45	+5	15, 35 **27, 27**	85	96.5	+13.5	0.52	0.59	0.07	
29	" 21	53	+5	10, 25, 43 **21, 21, 22**	92	102	+10.9	0.56	0.62	0.06	Same frog as in Experiment 28.
30	" 24	53	+5	11, 48 **33, 33**	84	91.5	+8.9	0.51	0.56	0.05	
31	" 24	47	+5	10, 43 **33, 43**	85	94	+10.6	0.52	0.57	0.05	" " " " " 30.
35	" 28	70	+2	10, 60 **37, 37**	84	87.5	+4.2	0.51	0.53	0.02	
36	" 28	50	+2	5, 40 **34, 36**	91.5	96	+4.9	0.56	0.58	0.02	" " " " " 35.
Average.....							+9.0			0.05	
Group B. Experiments not technically perfect.											
1	Feb. 18	75	+3 −15	18, 40, 53 **46, 40, 36**	98	95	−3.1	0.60	0.58	−0.02	Saline intravenously during collection.
8	Mar. 4	60	+3	15, 50 **33**	98	113	+15.3	0.60	0.69	0.09	Evaporation. Same frog as in Experiment 7.
11	" 7	43	+3	15, 40 **29, 33**	103	121	+17.5	0.63	0.74	0.11	Evaporation.
15	" 11	38	+3	13, 33 **39, 40**	97	110	+13.4	0.59	0.67	0.08	" Air bubble.
16	" 11	47	+3	15, 35 **38**	106	60.5	−42.9	0.65	0.36	−0.29	Same frog as in Experiment 15.
17	" 12	46	+3	14, 32 **31, 39**	95	101	+6.3	0.58	0.62	0.04	Plasma value uncertain.
20	" 14	73	+4	25, 50 **50**	100	102	+2.0	0.61	0.62	0.01	Cell not quite full. Same frog as in Experiment 19.

TABLE III—*Concluded.*

Experiment No.	Date.	Glomerular urine collection.		Blood.	Conductivities.			NaCl equivalents.			Notes.
		Time.	Pressure.	Time* / R. B. C. volume	Plasma.	Glomerular urine.	Difference.	Plasma.	Glomerular urine.	Difference.	
	Group B. Experiments not technically perfect—Concluded.										
	1929	*min.*	*mm. Hg*	*min.* / *per cent*	$K_{20°} \times 10^4$	$K_{20°} \times 10^4$	*per cent*	*gm. per 100 cc.*	*gm. per 100 cc.*	*gm. per 100 cc.*	
25	Mar. 20	57	−1	35 33	113	108	−4.4	0.69	0.66	−0.03	Cell not quite full. Tubule open. Same frog as in Experiment 23.
27	May 20	75	+5	30, 55 37, 39	95	97	+2.1	0.58	0.59	0.01	Plasma value uncertain.
32	" 24	50	+5	10, 25 29, 28	96	109	+13.5	0.58	0.67	0.09	Evaporation. Air bubble.
33	" 25	95	+5 +1	10, 80 29, 32	100	110	+10.0	0.61	0.67	0.06	Cell not quite full.
34	" 27	70	+2	0, 60 29, 30	96	102	+6.3	0.58	0.62	0.04	Air bubble.
37	" 29	46	+5	8, 58 34, 41	79.5	87	+9.4	0.48	0.53	0.05	Cell constant uncertain.
Average excluding Experiment 16							+7.4			0.04	

to yield a more reliable conclusion than those of Group B. In this group the range and the mean of the differences between glomerular urine and plasma are almost precisely the same as those found in the study of ultrafiltrates. Electrical conductivity of glomerular urine exceeded that of plasma by from 4.2 to 13.5 per cent; average 9.0 per cent. This correspondence is

TABLE IV.

Electrical Conductivity of Ultrafiltrates from Necturus Plasma.

Date.	Conductivity.			NaCl equivalents.			
	Plasma.	Ultra-filtrate.	Differ-ence.	Plasma.	Ultra-filtrate.	Differ-ence.	
1929	$K_{20°} \times 10^4$	$K_{20°} \times 10^4$	*per cent*	*gm. per 100 cc.*	*gm. per 100 cc.*	*gm. per 100 cc.*	
June 1	92.5	102	+10.3	0.56	0.62	0.06	After glomerular urine collection.
" 8	90	94	+4.4	0.55	0.57	0.02	" "
" 10	91.5	95.5	+4.4	0.56	0.58	0.02	" "
" 12	80	93	+16.3	0.48	0.56	0.08	" "
" 14	87	92	+5.7	0.53	0.56	0.03	" "
" 15	89.5	92	+2.8	0.545	0.56	0.015	" "
Average.............			+7.3			0.04	

strikingly shown if the differences, expressed as NaCl concentrations, are arranged in ascending order.

Difference between plasma and:	NaCl equivalents, gm. per 100 cc.												
Ultrafiltrate..	0.03	0.04	0.04	0.04	0.04	0.05	0.05	0.05	0.06	0.08			
Glomerular urine......	0.02	0.02	0.04	0.05	0.05	0.05	0.06	0.06	0.06	0.06	0.06	0.07	0.07

But one conclusion can be drawn from the results of this group; *viz.*, that the electrical conductivity of glomerular urine is indistinguishable from that of an ultrafiltrate.

In Group B are six determinations the results of which could find place in the former series without affecting the conclusion just stated. In the remaining seven the differences are greater and more variable. With two exceptions, Experiments 16 and 27, the nature of the fault in the determinations (see column "Notes")

TABLE V.

Electrical Conductivity of Blood Plasma and Glomerular Urine from Necturus Maculosus.

Experiment No.	Date.	Glomerular urine collection. Time.	Glomerular urine collection. Pressure.	Blood. $\frac{\text{Time*}}{\text{R. B. C. volume}}$	Conductivity. Plasma.	Conductivity. Glomerular urine.	Conductivity. Difference.	NaCl equivalents. Plasma.	NaCl equivalents. Glomerular urine.	NaCl equivalents. Difference.	Notes.
	Group A. Experiments with no known technical faults.										
	1929	*min.*	*mm. Hg*	$\frac{\textit{min.}}{\textit{per cent}}$	$K_{20^\circ} \times 10^4$	$K_{20^\circ} \times 10^4$	*per cent*	*gm. per 100 cc.*	*gm. per 100 cc.*	*gm. per 100 cc.*	
40	June 5	38	−2	48 44	95.5	98	+2.6	0.58	0.60	0.02	
41	" 5	27	−2	32 35	95.5	61	−36.1	0.58	0.36	−0.22	Same animal as in Experiment 40.
43	" 6	45	+3	50 14	93	99	+6.4	0.57	0.60	0.03	
46	" 10	30	+4	35 50	97	101	+4.1	0.59	0.62	0.03	
48	" 14	15	+1	20 30	87	90	+3.4	0.53	0.55	0.02	
49	" 14	27	+1	32 37	86	89.5	+4.1	0.52	0.54	0.02	Same animal as in Experiment 48.
50	" 14	22	+1	27	89	91	+2.3	0.54	0.55	0.01	" " " " " 48.

51	June 15		+		91.5	92.5	+1.1	0.56	0.56	0.00	
52	" 15	16	+3	26	89	93	+4.5	0.54	0.57	0.03	Same animal as in Experiment 51.
53	" 15	15	+3	25 **28**	88	91.5	+4.0	0.53	0.56	0.03	" " " " " 51.
Average excluding Experiment 41....................							+3.6			0.02	
Group B. Experiments not technically perfect.											
38	May 31	30	+(?)	35 **24**	85	100	+17.6	0.52	0.61	0.09	Evaporation. No urethane.
39	" 31	25	+(?)	30 **30**	93	118	+26.9	0.57	0.72	0.15	" " " Same animal as in Experiment 48.
44	June 7	35	+2	40	90	95	+5.6	0.55	0.58	0.03	Cell not quite full. No urethane.
45	" 8	36	+2	45 **24**	96	94	−2.1	0.58	0.57	−0.01	Plasma value uncertain.
47	" 12	70	+1	75 **46**	74.5	66	−11.4	0.45	0.40	−0.05	Saline intravenously during collection.
Average..							+7.3			0.04	

* "Time" of blood samples is expressed as minutes after beginning glomerular urine collection. Figures in bold-faced type are the hematocrit readings.

was such as to indicate the direction of the deviation from the mean which actually occurred. One of the exceptions, Experiment 16, is one in which no source of suspicion was recognized from the standpoint of technique; but the result is so utterly at variance with the rest as to make it incredible. A similar variant is contained in Walker's series of molecular concentration estimations and another among the phenol red estimations of Richards and Walker. The comment made by them (p. 491) is applicable here. It will be observed that the mean of the glomerular urine conductivity estimations in Group B of Table III is almost exactly the same as those of Group A and of the ultrafiltrate series, and that the distribution of values on either side of the mean is approximately equal. No reason is to be found in these aberrant results for questioning seriously the validity of the conclusion drawn from the more perfect experiments.

Ultrafiltrates from Necturus Plasma.—Six comparisons of electrical conductivity of *Necturus* plasma and ultrafiltrate from it are given in Table IV. In each case the blood was taken from an animal which had already served in a collection of glomerular urine.

Glomerular Urine from Necturus.—Fifteen comparisons of electrical conductivity of glomerular urine and blood plasma are available in Table V. As in Table III, we have separated the data into two groups: ten experiments in which no technical fault was noted (Group A) and five experiments in which some mishap occurred (Group B). The agreement between the results of Group A and the ultrafiltration experiments (Table IV) is not as exact as in the experiments on frogs; but since this is due to the greater variability in the conductivities of ultrafiltrates it has less significance than has the consistency of the values for glomerular urines.

Difference between plasma and:	NaCl equivalents, gm. per 100 cc.								
Ultrafiltrate	0.015	0.02	0.02	0.03	0.06	0.08			
Glomerular urine	0.00	0.01	0.02	0.02	0.02	0.03	0.03	0.03	0.03

Experiment 41 presents an exceptional result similar to that of frog Experiment 16. In this case we have a definite and satis-

factory explanation for it. The glomerular urine was sucked from the capsule and during the collection the degree of suction applied was intermittently increased. This circumstance forces the belief that in this instance fluid was collected which had passed from the capsule of Bowman into the tubule and was drawn back into the pipette after a considerable fraction of its NaCl had been reabsorbed.

The comments which were made upon the experiments of Group B in Table III apply equally to Group B in Table V. Consideration of the nature of the errors and of the degree and sign of the variations observed shows clearly that the variations are technical rather than physiological.

TABLE VI.

Results of Estimations of Electrical Conductivity and Total Molecular Concentration on the Same Specimens of Glomerular Urine and Plasma.

Experiment No.	Animal.	Molecular concentration.*	Electrical conductivity, percentage difference from plasma.	Experiment No. in Walker's paper.
18	Frog.	+1	+11.2	21
16	"	−24.6	−42.9	19
40	*Necturus.*	+0.6	+2.6	7
49	"	+1.7	+4.1	11
41	"	−21.1	−36.1	8

* Figures in this column are percentage change in volume of glomerular urine after equilibration for 48 hours against plasma in a Barger tube. Values of 1.5 per cent or less indicate identity.

In Tables III and V in this paper and in Tables III and V in the preceding paper by Walker there are a number of experiments in each of which electrical conductivity and total molecular concentration were measured on the same collection of glomerular urine and plasma. In five of these both determinations were believed to be technically perfect. The results are shown in Table VI: they exhibit an agreement which adds to our confidence in the reliability of the methods as used and in the correctness of the conclusion drawn.

The experiment of March 12 (Experiment 17, Table III of this paper; Experiment 20, Table III of the preceding paper by Walker) is worthy of especial mention since not only were the plasma speci-

mens and glomerular urine analyzed both by determination of the total molecular concentration and of the electrical conductivity, but measurements were also made of the phenol red concentration, a subcutaneous injection having been made at the beginning of the experiment. At the end of the experiment, also, a sample of the plasma was taken and ultrafiltered, measurements of electrical conductivity, total molecular concentration, and phenol red concentration being made on the ultrafiltrate and plasma from which it was derived. They are presented below.

	Difference from plasma with respect to:		
	Phenol red concentration.	Total molecular concentration.	Electrical conductivity.
	per cent		*per cent*
Ultrafiltrate........................	33	0	6.0
Glomerular urine..................	37	0	8.5

SUMMARY.

A cell has been designed which enables accurate estimation of electrical conductivity of fluids in volume as small as 0.5 c.mm.

Comparisons have been made between the electrical conductivities of blood plasma and ultrafiltrates and between those of blood plasma and glomerular urine obtained from the frog and from *Necturus maculosus*.

The conclusion which these comparisons yield is that in respect of total electrolyte concentration the glomerular urine from both species is indistinguishable from an ultrafiltrate of plasma.

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ON THE MICRO DETERMINATION OF LIPIDS IN TISSUES.

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The determination of the amount of fatty acid in organ tissue has been attempted by not a few investigators. Dormeyer (13), Rosenfeld (30), Bogdanow (10), Frank (18), Nerking (27), Liebermann and Székely (24), Voit (38), Glikin (20), and Sasaki (31) have endeavored to get the total possible extract with adequate solvent and by proper treatment of material. Having carried out a precise analysis of the subject, Kumagawa and Suto (23) pointed out in 1908 that extracts obtained by the investigators mentioned contained some impurity and moreover that not a small percentage of higher fatty acid had escaped from extraction. The Kumagawa-Suto method has since been considered the standard for determination of fatty acid in tissue. Shimidzu (32), a student of Kumagawa, proved that the method was also available in the case of organ tissues other than skeletal muscle, *viz.* the liver, the kidney, and the heart muscle. Watanabe (39) applied this method to fresh tissues with equally good results. Neutral fat was calculated by Kumagawa and Suto by multiplying the weight of fatty acid by a factor 1.046, presuming that solid and liquid fatty acids are contained in a constant proportion in the muscle lipid. Zelinsky and Zinzadge (41) have recently digested tissue with sulfuric acid or hydrochloric acid in an autoclave. The protein was decomposed and the fat was half hydrolyzed. Then the fatty substance was extracted and purified by the Kumagawa-Suto method and weighed. Next they computed the amount of free fatty acid from the acidity of the extract. The amount of glycerol corresponding to the free fatty acid was calculated and added to the weight of the fatty substance, the sum thus obtained giving the true amount of total neutral fat.

As to the quantitative determination of cholesterol there are the colorimeteric (the Liebermann-Burchard reaction), the spectrochemical (Lifschütz (25)), as well as the gravimetric method. Windaus' (40) method of gravimetric determination with digitonin is regarded as the best of all. In the application of this method to tissue cholesterol Windaus mixed minced tissue with plaster of Paris, and dried and reduced it to a powder from which the fatty substance was extracted by means of alcohol for a

period of 3 to 4 days. In this extract he measured the free as well as the combined form of cholesterol. Windaus' method was tested and modified by later investigators (Fraser and Gardner (19), Thaysen (36), Fex (17), Müller (26), and Onizawa (28)) to a much more exact procedure. The colorimetric method was used by Autenrieth and Funk (1), Bloor and coworkers (9), Embden and Lawaczeck (15), and Takahata and Nakamura (35).

As for the determination of phospholipids in tissue, Costantino (12) has extracted tissue with a Soxhlet apparatus, in which extract the amount of lipid P was estimated. Kumagawa and Suto have modified the Soxhlet apparatus, so that the extraction is complete. They calculated the phospholipid also, multiplying the measured phosphorus of lipid by a certain factor. Recently Bloor (8) has reported that his oxidative method of determination of fatty acid is applicable to the determination of phospholipids.

For the micro determination of lipids in blood Bang (4), Bloor and coworkers (5, 9) and Takahata and Nakamura (35) have reported different methods. Bloor's method of determination of blood lipids is used widely because of its simplicity.

In the present report we hope to demonstrate a simple and complete method for the extraction of tissue lipids for their micro determination without using any strong chemical reagent such as sodium hydroxide, or a high temperature, processes which are inevitable to some extent in the methods of Kumagawa and Suto, Fex, and Onizawa, and which may injure some sensitive lipids such as lecithin and cephalin. So that the solvent could touch the substance to be extracted as well as possible, tissue and cells were destroyed thoroughly by freezing, moistening, and grinding in the mortar. This new method is not new. It was used lately by Avery and his associates (2) for obtaining carbohydrate from bacteria cells and by Embden and his students (21) for extracting inorganic phosphate, lactacidogen, and lactic acid from tissue. But no one has applied it to the extraction of tissue lipids, as far as we know. We published our preliminary report in January, 1927, in the 64th assembly of the Kanazawa Medical Association (29). Subsequent to this report Endo (16), from the biochemical institute here, has proved the availability of our procedure and modified it by the use of fine sand and a mortar for braying the tissue. In February, 1929, Bloor (8) reported a similar method of destroying tissue for extracting phospholipids from tissue. As to the solvent we first used Bloor's (9) alcohol-ether mixture (3:1) and

then alcohol-ether-chloroform mixture (3:1:1). Now we prefer alcohol-ether mixture (1:1) as Endo reported.

Extraction.

About 0.5 to 2.0 gm. (if necessary 5.0 to 10.0 gm. of material may be used with equally good results) of tissue are weighed accurately on a fragment of test-tube glass and put in a mortar of 8 to 9 cm. diameter immersed beforehand in a freezing mixture. (We use for convenience a mixture of ice and salt. The use of liquid air which Embden used would be better.) In the course of freezing and moistening the tissue is ground with a pestle. The tissue changes very easily into a homogeneous paste. The glass fragment ground together with the tissue and reduced to a powder helps the braying of the tissue. A few cc. of water are added and freezing, moistening, and grinding are repeated once more. About 10 cc. of alcohol-ether mixture (3:1) are added and mixed well. The whole content of the mortar is transferred into an Erlenmeyer flask by means of a glass rod. The adherent material is rinsed repeatedly with portions of alcohol-ether mixture (1:1) and collected in the flask, until the volume is about 50 cc. The flask may be heated to gentle boiling on the water bath. The heating is not absolutely necessary, if the flask is allowed to stand for several hours at room temperature and its contents stirred at intervals. The contents of the flask are decanted and the supernatant solvent is filtered into a 100 cc. volumetric flask through fat-free filter paper. About 20 cc. of the alcohol-ether mixture (1:1) are added to the Erlenmeyer flask and stirred for several minutes and filtered through the same filter paper. The Erlenmeyer flask and the filter paper are rinsed repeatedly with the alcohol-ether mixture, care being taken not to allow the tissue residue to fall into the volumetric flask. This is done until the volumetric flask is full to the mark, at room temperature.

EXPERIMENTAL.

The experiment was performed with the following points in view: (1) To examine whether the extraction of lipids in tissue with our procedure is complete; and (2) to check the availability of our procedure for the micro determination of lipids in tissue.

1. The Petroleum Ether-Soluble Substance.

Minced muscle of beef was treated with sodium hydrochloride according to Kumagawa and Suto (23) and its petroleum ether-soluble substance was gravimetrically determined. The extract

TABLE I.

Petroleum Ether-Soluble Substance of Minced Beef Muscle.

Experiment No.	Weight of muscle.	Extraction method.	Petroleum ether-soluble substance in extract.		Petroleum ether-soluble substance in tissue residue.	
				Per cent in relation to Kumagawa-Suto average.		
	gm.		*gm.*		*gm.*	*per cent*
1	1.0	Kumagawa-Suto.	0.0176	96.9		
2	1.0		0.0186	102.1		
3	1.0		0.0184	101.1		
Average			0.0182	100.0		
1	1.0	Osato-Heki.	0.0173	95.0	0.00	0.0
2	1.0		0.0177	97.2	0.00	0.0
3	1.0		0.0196	107.6	0.00	0.0
Average			0.0182	100.0	0.00	0.0
1	1.0	Kumagawa-Suto.	0.0108			
2	1.0		0.0109			
3	1.0		0.0108			
Average			0.0108	100.0		
1	1.0	Osato-Heki.	0.0108		0.00	0.0
2	1.0		0.0108		0.00	0.0
3	1.0		0.0108		0.00	0.0
Average			0.0108	100.0	0.00	0.0

obtained by our procedure was prepared from the same material and it was saponified with sodium hydroxide, the ether of the solvent being evaporated beforehand. Then its petroleum ether-soluble substance was weighed. The tissue residue of our extract was treated with sodium hydroxide and its petroleum ether-

soluble substance was determined by the procedure of Kumagawa and Suto. Several experiments, which have presented almost the same results, are given in Table I. Similar determinations on 5 gm. of material gave results equally good. Extraction is almost complete with 1.0 gm. of tissue as well as with 5.0 gm.

The result was the same with some other organ tissues: liver,

TABLE II.

Petroleum Ether-Soluble Substance in Various Organ Tissues.

Kind of tissue.	Experiment No.	Weight of material.	Petroleum ether-soluble substance. In extract.	Petroleum ether-soluble substance. In tissue residue.	Total petroleum ether-soluble substance.	Petroleum ether-soluble substance of tissue residue in total petroleum ether-soluble substance.
		gm.	*gm.*	*gm.*	*gm.*	*per cent*
Liver.	1	0.5	0.0151	0.0000	0.0151	0.0
	2	0.5	0.0161	0.0002	0.0163	1.2
	3	0.5	0.0158	0.0002	0.0160	1.2
Average			0.0157	0.0001	0.0158	0.8
Lung.	1	1.0	0.0131	0.0002	0.0133	1.5
	2	1.0	0.0130	0.0002	0.0132	1.5
	3	1.0	0.0139	0.0002	0.0141	1.3
Average			0.0133	0.0002	0.0135	1.4
Kidney.	1	1.0	0.0177	0.0001	0.0178	0.6
	2	1.0	0.0180	0.0000	0.0180	0.0
	3	1.0	0.0182	0.0001	0.0183	0.6
Average			0.0180	0.0001	0.0181	0.4

In this experiment alcohol-ether mixture (3:1) was used as the solvent. The figures in the sixth column were not checked by the Kumagawa-Suto method.

lung, and kidney (Table II). In no case did the petroleum ether-soluble substance remaining in the tissue residue exceed 1.5 per cent of that of the extract.

2. Application of Bloor's Method of Micro Determination of Blood Lipids to Tissue Materials.

The method of lipid determination in blood plasma as originally published by Bloor and modified afterwards by him and his

collaborators, has been used widely because of its simplicity. It was examined by us to find out whether the same principle holds good for the tissue extract obtained by our procedure. Bloor's older methods were tried out on material obtained by both the Kumagawa-Suto and the Osato-Heki procedures and it was found that while the cholesterol determination was quite satisfactory, the values for fatty acids by the nephelometric procedure were usually very low. In the case of blood Bloor has announced that the melting point of fatty acid mixture is fairly constant for a number of animals. Some tissue fatty acid might have had a melting point unsuitable for the use of Bloor's standard fatty acid mixture. But so far as we ascertained from some fatty acid preparation obtained from our tissue materials they had a slightly lower melting point. Up to date we have not been able to find the reason why we usually have obtained lower values for fatty acid by nephelometric methods than with gravimetric procedures. It is obvious that the cause does not lie in the different procedure of extraction, because with the same determination method the data of both the Kumagawa-Suto and the Osato-Heki extracts are in quite good agreement.

3. Application of the Bang-Bloor Oxidative Method for the Determination of Fatty Acid to the Tissue Extract.

Recently Bloor (7) has reported an improved method of Bang's (3) oxidative determination of fatty acid suitable for the micro determination of fatty acid in blood plasma. We performed many determinations on a solution of purified fatty acid derived by the Kumagawa-Suto method from a number of tissue materials and obtained results showing good agreement between gravimetric and oxidative methods. In the accompanying tabular matter some of our determinations are given. The data are calculated for 100 cc. of the solutions.

Kind of tissue from which fatty acid was derived.	Liver.	Kidney.	Lung.	Muscle.
	gm.	*gm.*	*gm.*	*gm.*
Gravimetric method....................	0.0385	0.0536	0.0408	0.0098
Oxidative "	0.0355	0.0513	0.0396	0.0094

Many determinations with the Bang-Bloor method were carried out on tissue extract obtained by the Osato-Heki procedure. As a check experiment macro determination of fatty acid and non-saponifiable substance on the same material was made with the Kumagawa-Suto method. The results are shown in Table III. It should be noted here that, as Kumagawa and Suto mentioned, the amount of non-saponifiable substance is not equal to cholesterol but to the sum of cholesterol and unknown non-saponifiable substance. If the value of cholesterol estimated colorimetrically is subtracted from the petroleum ether-soluble substance determined by Bloor's oxidative method, the unknown

TABLE III.

Determination of Fatty Acids and Non-Saponifiable Substance of Various Tissue Extracts.

Kind of tissue.	Experiment No.	Kumagawa-Suto extraction method. Gravimetric determination.			Osato-Heki extraction method. Oxidative and colorimetric, the Bang-Bloor method.		
		Weight of material.	Total fatty acid.	Non-saponifiable substance.	Weight of material.	Total fatty acid.	Cholesterol.
		gm.	*per cent*	*per cent*	*gm.*	*per cent*	*per cent*
Liver.	1	5.0	2.38	0.32	0.5	2.34	0.27
	2	5.0	1.87	0.32	0.5	2.15	0.24
	3	5.0	3.12	0.35	0.5	3.50	0.24
Kidney.	1	5.0	1.36	0.36	1.0	1.08	0.40
	2	5.0	1.12	0.41	1.0	1.12	0.34
	3	5.0	3.54	0.28	1.0	3.75	0.30
Lung.	1	5.0	1.54	0.48	1.0	1.44	0.45
	2	5.0	1.00	0.57	1.0	0.92	0.42
	3	5.0	1.04	0.31	1.0	1.25	0.30
Heart muscle.	1	6.0	1.44	0.11	1.2	1.33	0.11
	2	6.0	0.97	0.51	1.2	1.47	0.08
Skeletal muscle.	1	8.0	4.61	0.15	1.6	4.06	0.05
	2	8.0	0.41	0.08	1.6	0.50	0.04

Each experiment was carried out on different material at a different time.

non-saponifiable substance must be included in the remainder which is shown as fatty acid in the determination by the Bang-Bloor method. For this reason it is easily comprehensible, why the value of fatty acid obtained by the Bang-Bloor method does not quite agree with that obtained by the Kumagawa-Suto method. A better result would be obtainable, if all the fatty acid of the extract could be collected and the determination made on it. In any case the Bang-Bloor method is readily applicable to the tissue extract obtained by our procedure with much better results than with nephelometric methods.

4. Separate Determination of Free and Combined Cholesterol in Tissue by the Digitonin Method.

In a further investigation we proved that the digitonin method of Windaus, which Fraser and Gardner, Thaysen, von Szent-Györgyi (34), Tominaga (37) Müller, and Onizawa have extensively studied, is very suitable for separate micro determination of free and combined cholesterol in the tissue extract obtained by the Osato-Heki method. A slight improvement with a similar procedure, as Caminade's (11), to facilitate the precipitation of cholesterol digitonide shortens the time of operation and renders its application much easier. Details will be reported by one of us (Heki) later.

Free Cholesterol.—Aliquots of the extract (corresponding to 1 to 3 mg. of free cholesterol) are evaporated to dryness. The residue is extracted with portions of pure acetone which is filtered through a small funnel stuffed with fat-free cotton. The total volume of solvent is made up to 8 cc., and 2 cc. of distilled water are added. After warming the flask gently to about 60° on the water bath, 1 to 1.5 cc. of alcoholic solution of digitonin are added. Several minutes bring the reaction to the end. In the case of some tissue extract as suprarenal gland which contains much ester form of cholesterol it happens always that the acetone solution becomes turbid on the addition of 2 cc. of water. But it does not interfere with the operation. This turbidity, which is caused by precipitated cholesterol ester, may be dissolved by the addition of a few cc. of ether after the free cholesterol is precipitated with digitonin. After the precipitate is decanted the supernatant fluid is poured into a small asbestos funnel (Fig. 1). The precipitate is gathered

with portions of pure acetone on the same asbestos funnel, washed with acetone, then with ether, dried in an electric oven at 105° to a constant weight, and weighed accurately. The weight of cholesterol digitonide multiplied by the factor 0.2431 gives the weight of cholesterol.

Total Cholesterol.[1]—Aliquots of the same extract are measured

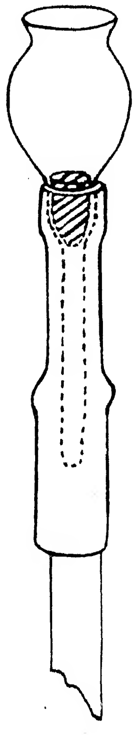

FIG. 1. The asbestos funnel (natural size) which is adjusted to a filter flask is the type used for collecting the cholesterol digitonide precipitate.

into a flask fitted to the return condenser and freed from ether. The volume of the remaining alcohol is reduced to 10 cc. 2 cc. of sodium ethylate solution (2 gm. of metallic sodium dissolved in

[1] We have carried out many check determinations on solutions with known concentration of cholesterol ester, which we prepared as Hürthle (22) described.

100 cc. of absolute alcohol) are added and boiled under a reflux condenser for 2 hours. After being cooled to the room temperature, the contents of the flask are transferred into a separatory funnel. The flask is rinsed with 30 cc. of ether which is also poured into the funnel. The alcoholic solvent and ether are mixed well. 20 cc. of water are added and shaken repeatedly. The under layer is discarded. Now the ether solution is washed twice with water, 10 cc. for each time. When the ether becomes opaque here, it may be clarified by shaking the funnel after the

TABLE IV.

Precipitation of Cholesterol Digitonide in 1 to 3 Mg. of Cholesterol.

Cholesterol taken.	Weight of cholesterol digitonide.	Cholesterol recovered.	
mg.	*mg.*	*mg.*	*per cent*
1.0	4.2		
1.0	4.0		
1.0	4.1		
Mean...........	4.1	0.99	99.7
2.0	8.2		
2.0	8.3		
2.0	8.2		
Mean...........	8.2	1.99	99.7
3.0	12.3		
3.0	12.3		
3.0	12.4		
Mean...........	12.3	3.00	100.0

addition of a few drops of saturated watery solution of NaCl. The ether solution is then taken into a small flask and freed from the solvent by evaporation. The residue is dissolved in 8 cc. of pure acetone, which is treated in the same way as in the determination of free cholesterol. The combined cholesterol may be calculated by subtracting free cholesterol from the total cholesterol.

8 cc. of pure acetone mixed with 2 cc. of water do not precipitate until 8 mg. of pure cholesterol are dissolved in it. 1 to 3 mg. of cholesterol, with which we are concerned, do not interfere with

the operation. How complete the precipitation of cholesterol digitonide is, is shown in the following examples, in which the estimation was carried out on a solution of pure cholesterol (Table IV) as well as on a tissue extract with a known amount of cholesterol added (Table V).

TABLE V.

Precipitation of Cholesterol Digitonide in Tissue Extract with Added Cholesterol.

Substance tested.	Weight of cholesterol digitonide.	Cholesterol found.	Recovery of added cholesterol.
	mg.	*mg.*	*mg.*
Liver, 0.5 gm.	3.9		
	4.1		
	4.0		
Mean...............	4.0	0.97	
0.5 gm. liver + 1.0 mg. cholesterol.	8.2		
	8.2		
	8.3		
Mean...............	8.2	1.99	1.02

5.0 gm. of liver were extracted with 100 cc. of alcohol-ether-chloroform mixture (3:1:1) by our procedure. A 10.0 cc. aliquot (corresponding to 0.5 gm. of liver) was taken for each experiment. Cholesterol was added as a solution in chloroform. The determination was the same as for free cholesterol described above.

5. Examination of Tissue Residue in Our Extraction Procedure with Regard to Cholesterol Content Which May Have Remained Unextracted.

Examination of the tissue residues by saponification after the Osato-Heki extraction gave only unweighable traces of precipitate with digitonin, showing that the cholesterol had been completely extracted.

6. Determination of Phospholipids in the Tissue Extract.

Tissues of beef used in these experiments were taken from the slaughter-house as soon as the animal was killed. Samples of tissue were weighed accurately and extracted by the Osato-

Heki method. The extract was made up to 100 cc. Of the same material a known amount of minced tissue was placed in a brown glass desiccator over $CaCl_2$ under diminished pressure. 1 to 2 days are sufficient for desiccation. The dried tissue was then reduced to a powder in a mortar. An amount of fried tissue powder exactly corresponding to the weight of fresh material taken for the Osato-Heki procedure was subjected to the Kumagawa-Suto (23, 33) method of hot extraction with absolute alcohol. The resultant extract was also made up to 100 cc. 50 cc. of each of the extracts were measured into a long necked Kjeldahl flask and evaporated to dryness under diminished pressure with the aid of a water pump on the water bath. The residue was dissolved in pure ether and filtered through an asbestos funnel into a Kjeldahl flask. The phosphorus was determined by Embden's (14) method. In experiments on muscle, which contains usually a small amount of phospholipids, the amount of sample used was not enough for the optimal phosphorus content, 1 to 4 mg. of P_2O_5, of Embden's method. From our experiences in many control determinations we are convinced that the method gives good results with a smaller amount of P_2O_5, if the weighing is done cautiously with a sufficiently sensitive balance. The tissue residue in the Osato-Heki procedure was reduced to a powder and extracted in the Kumagawa-Suto apparatus, on which extract the same operation as above was performed to determine its phosphorus content. In the experiment on muscle (Table VI) aliquots of the extract (20 cc. in this case) were taken and the phosphorus content was determined by Bloor's nephelometric method (5).

From Tables VI and VII it is obvious that the extraction of phospholipids by our procedure is also complete. The largest remaining phospholipid content in the tissue residue of liver does not exceed 1.5 per cent, which can be practically neglected. So far as we experienced the measurement of the small amount of phospholipids is very accurate by Embden's method, if the weighing is done carefully. Of course, the nephelometric or colorimetric method of phosphorus determination is also applicable, if great accuracy is not demanded (Table VI). For the determination of phospholipids in blood Bloor (5) has calculated it from the amount of phosphorus, measured directly on the alcohol-ether extract of

blood. But in the case of tissue phospholipids we prefer to reextract the tissue extract obtained by our procedure, as we described it above, because a small amount of other kinds of phosphorus

TABLE VI.

Determination of Phosphorus in Tissue Extracts.

Kind of tissue.	Extraction method.	Experiment No.	Weight of material.	Phospholipid P determined gravimetrically (Embden's method). In extract.	In tissue residue.	In tissue residue. Lipid P in extract.	Phospholipid P determined nephelometrically (Bloor's method).
			gm.	*mg.*	*mg.*	*per cent*	*mg.*
Muscle.	Kumagawa-Suto.	1	1.0	0.248			0.25
		2	1.0	0.26			
		3	1.0	0.275			
		4	1.0	0.244			0.25
		Average		0.257			0.25
	Osato-Heki (warm).	1	1.0	0.268	0.000	0.00	0.25
		2	1.0	0.253	0.009	0.32	
		3	1.0	0.251	0.000	0.00	0.25
		Average		0.257	0.003	0.11	0.25
	Osato-Heki (cold).	1	1.0	0.275	0.008	0.31	0.25
		2	1.0	0.249	0.000	0.00	
		3	1.0	0.251	0.000	0.00	0.24
		Average		0.258	0.003	0.1	0.25
Liver.	Kumagawa-Suto.	1	1.0	1.766			
		2	1.0	1.744			
		3	1.0	1.823			
		Average		1.778			
	Osato-Heki (warm).	1	1.0	1.758	0.022	1.27	
		2	1.0	1.764	0.010	0.56	
		3	1.0	1.787	0.025	1.38	
		4	1.0	1.756	0.027	1.51	
		5	1.0	1.797	0.026	1.47	
		Average		1.772	0.022	1.24	

than phospholipid P may very probably be contained in the primary extract as is obvious from Table VII.

Experiments with larger amounts of material (5 to 10 gm.), which are not presented in this report, were repeated for fractions of lipids on the above mentioned organs and nearly the same results were obtained.

In the practical determination 0.5 to 2.0 gm. of tissue are suffi-

TABLE VII.

Determination of Phospholipid Phosphorus of Extracts of Various Tissue Extracts.

Kind of tissue.	Extraction method.	Weight of material.	Phospholipid P determined gravimetrically by Embden's method. In extract. Alcohol-ether-soluble.	In extract. Ether-soluble.	In tissue residue.	In tissue residue. Lipid P in extract.
		gm.	*mg.*	*mg.*	*mg.*	*per cent*
Spleen.	Kumagawa-Suto.	1.0		0.695		
	Osato-Heki.	1.0	0.845	0.725	0.00	0.0
Kidney.	Kumagawa-Suto.	1.0		0.882		
	Osato-Heki.	1.0	0.905	0.876	0.00	0.0
Lung.	Kumagawa-Suto.	1.0		0.754		
	Osato-Heki.	1.0	0.831	0.802	0.012	0.1
Heart muscle.	Kumagawa-Suto.	1.0		0.946		
	Osato-Heki.	1.0	0.933	0.927	0.021	0.2

The alcohol-ether-soluble P determinations of the Osato-Heki extract (fourth column) were made directly on 30 cc. of the extract without reextracting with ether.

cient to carry out a duplicated estimation of all lipids for most of the organs. But in the case of skeletal muscle, which usually contains a far smaller amount of lipids, 2.0 to 4.0 gm. of material must be taken, so that a sufficient content of lipids in the extract can be obtained.

The results of lipid determination in the case of five male rabbits are given in Table VIII. The individual variation in the con-

TABLE VIII.

Lipid Content of Various Organs of the Rabbit.

The lipid fraction values are given in per cent and the weight of the organs in gm.

		Blood.	Liver.	Spleen.	Kidney.	Lung.	Heart muscle.	Skeletal muscle.
Rabbit 1. 2.28 kg.; male; white.	Weight of organ.		43.0	0.69	14.1	7.1	6.0	
	Total cholesterol.	0.096	0.212	0.264	0.377	0.460	0.085	0.032
	Free "	0.046	0.065	0.123	0.240	0.377	0.040	0.021
	Fatty acid.	0.27	3.13	3.00	2.70	3.10	2.12	0.47
	Lecithin.	0.17	2.79	1.61	1.97	2.23	2.15	0.40
Rabbit 2. 2.16 kg.; male; white.	Weight of organ.		45.0	2.6	14.3	8.3	5.8	
	Total cholesterol.	0.074	0.200	0.195	0.265	0.437	0.105	0.040
	Free "	0.058	0.188	0.195	0.265	0.437	0.105	0.040
	Fatty acid.	0.24	3.12	1.87	2.97	3.62	1.90	0.76
	Lecithin.	0.24	3.39	1.99	2.53	2.23	1.91	0.56
Rabbit 3. 2.68 kg.; male; white.	Weight of organ.		45.0	1.0	15.2	8.2	6.0	
	Total cholesterol.	0.062	0.248	0.300	0.325	0.450	0.108	0.034
	Free "	0.042	0.211	0.300	0.325	0.450	0.108	0.034
	Fatty acid.	0.22	3.60	2.22	2.55	2.70	2.33	0.54
	Lecithin.	0.26	3.38	1.51	2.41	3.69	1.75	0.46
Rabbit 4. 2.71 kg.; male; white.	Weight of organ.		47.0		16.3	11.5	7.0	
	Total cholesterol.	0.060	0.230		0.375	0.475	0.127	0.063
	Free "	0.050	0.225		0.340	0.475	0.127	0.063
	Fatty acid.	0.35	3.45		3.02	3.37	2.50	0.58
	Lecithin.	0.17	3.43		2.92	2.53	1.83	0.47
Rabbit 5. 1.95 kg.; male; white.	Weight of organ.		33.5	0.6	12.3	9.3	5.3	
	Total cholesterol.	0.094	0.352	0.274	0.304	0.400	0.104	0.042
	Free "	0.079	0.304	0.163	0.223	0.340	0.075	0.061
	Fatty acid.	0.18	3.25	1.08	3.00	2.15	2.34	0.66
	Lecithin.	0.21	2.95	1.97	2.61	3.10	1.91	0.42

Phospholipid P was estimated by Bloor's nephelometric method and is given as lecithin.

tents of the fractions of lipids in each organ is not very large. Cholesterol of organ tissue consists for the most part of the free form. The contents of cholesterol ester in tissue are inconstant and small. Sometimes it is absent in tissue, as Bloor has reported recently.

SUMMARY.

1. In the present report a simple method for extraction of tissue lipids is described.

2. Its availability for the micro determination of the fractions of tissue lipids is discussed.

3. A convenient procedure for the separate micro determination of free and combined cholesterol is given.

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THE JANSEN AND DONATH PROCEDURE FOR THE ISOLATION OF ANTINEURITIC VITAMIN.

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Early in 1927, the attention of students of vitamin chemistry was caught by the appearance of an unusual paper by Jansen and Donath (1) of the Medical Laboratory, Weltevreden, Java. The purport of the paper was that by means of a somewhat complicated procedure, based largely on the use of reagents previously suggested by Funk and Seidell, a pure crystalline substance of antineuritic properties had been obtained of the composition $C_6H_{10}N_2O \cdot HCl$. There are many evidences in the paper of careful and painstaking work and the course of fractionation seems reasonable and roughly in harmony with past experience. It was felt that their claims deserved serious and deliberate consideration.

The physiological testing necessary as a guide in their procedure involved the use of rice birds and of a special feeding technique. As neither the experimental animals nor the technique has been used by other workers it is difficult to correlate the Dutch workers' results with those of older literature. The best means of doing so lay in the fact that in the case of their final product Jansen and Donath tested the effect of the material upon a few pigeons. The result was complete prophylaxis against polyneuritis, and a high degree of maintenance of appetite but accompanied by moderate losses in weight, except when polished rice was supplemented with extracted meat powder to improve the protein of the diet. In this case no significant loss of weight occurred.

On account of the weight losses we were once inclined to doubt that the isolated crystals were the long sought antineuritic vitamin,

which has been generally credited with the power to maintain the weight of pigeons on a polished rice diet as well as protect against polyneuritis. However, this consideration presently came to have a favorable rather than an unfavorable significance when we discovered evidence of the existence of a "third factor" (2) necessary to weight maintenance in pigeons. More or less of this third factor may be supplied by cod liver oil or meat powder which Jansen and Donath used as supplements to polished rice.

In view of the apparent importance of Jansen and Donath's contribution we determined to repeat their chemical work in detail and to carry out extended feeding tests upon both rats and pigeons not only with the final product but also with each of the principal fractions discarded.

In order to become familiar with the method it was decided first to apply it to yeast extract which we had immediately available and to substitute fullers' earth for the acid clay used by Jansen and Donath. In the meantime steps were taken to secure supplies of rice polish and acid clay and to fit up suitable apparatus for the extraction process as described in the original paper.

Yeast Experiments.

170 kilos of moist pressed yeast (approximately 50 kilos of dry weight) were extracted for us by the Harris Laboratories, Tuckahoe, N. Y., by means of hot acidulated water after the manner of Osborne and Wakeman. The filtered extract was concentrated *in vacuo* to about 60 liters volume, brought to pH 4.5 by addition of sulfuric acid, and stirred for 4 hours with 1500 gm. of fullers' earth which had passed a 200 mesh sieve. The fullers' earth was filtered off and submitted to fractionation by Jansen and Donath's method with only such minor modifications as are indicated below.

Products at various stages of fractionation were tested as supplements to polished rice as a diet for pigeons. They were also used as supplements for Sherman and Spohn's basal Diet 107 (3) in the nutrition of young rats over the usual 60 day feeding period. Autoclaved yeast was used as an additional supplement in the rat experiments. In both the pigeon and rat experiments a quantitative evaluation of each fraction was attempted by means of one or more trial readjustments of dosage. Necessarily the

quantitative estimation of activity involved a considerable element of personal judgment, as it soon became evident that qualitative as well as quantitative differences existed among the several fractions. The results are recorded in schematic form below, and the quantitative aspects of the matter are indicated by the number of doses appearing in each fraction. For this purpose a dose is defined as the daily quantity which had to be administered to a rat or pigeon previously depleted of vitamin B_1, in order to produce maximum physiological benefit, without exceeding by more than a factor of 3 the quantity which would just produce a distinct benefit. The reason for such a definition is that in many cases it was found that increasing the dose over a certain range markedly improved the weight level or rate of growth, while further increases in dosage resulted in much smaller effects which appeared to be due to the presence of lesser amounts of some factor other than the antineuritic vitamin. Our daily dose is thus intended to correspond roughly to the point of maximum inflection of a curve in which increasing doses are plotted against increase of weight. In all cases this dose somewhat exceeded that necessary to protect against outright symptoms of polyneuritis. It should perhaps be said explicitly that the estimate of doses may be in error by a factor of as much as 2 in any given case.

It should also be remarked that the experiments with rats were probably somewhat complicated by the inavoidable presence of traces of antineuritic vitamin in the autoclaved yeast (4). These traces were however insufficient to obscure the supplementary relation between the autoclaved yeast and the products of the Jansen and Donath fractionation. Fortunately the evidence is very convincing that only traces of vitamin B_2 accompany the antineuritic vitamin in the fullers' earth adsorption process, and negligible quantities appear in any fraction beyond that point.

The schematic distribution of activity is indicated in Diagram 1.

In the above silver nitrate fractionation each fractional precipitate was dissolved in acid and again fractionally precipitated with silver nitrate and baryta. This was the interpretation we then placed upon Jansen and Donath's description of their procedure. In treating the platinum chloride precipitate we intentionally departed from the procedure by separating the water-soluble from the water-insoluble portion of this precipitate because only about

DIAGRAM 1.

Yeast extract treated with 1800 gm. of fullers' earth

- Filtrate 20,000 doses r.*
- Fullers' earth {100,000 r. 150,000 b.
  - Treated with $Ba(OH)_2$
    - Residue {5000 r. 10,000 b.
    - Extract
      - Fractionated with $AgNO_3 + Ba(OH)_2$
        - Precipitated to pH 4.5
          - Fraction S113 {500 r. 1000 b.
          - Fraction S115 1000 b.
        - Precipitated to pH 6.5
          - Decomposed with HCl and treated with phosphotungstic acid
            - Filtrate after removal of phosphotungstic acid
              - Fraction S108 {<500 r. 2000 b.
            - Ppt.
              - Dissolved in acetone and reprecipitated
                - Filtrate
                  - Fraction S112 {<100 r. 2000 b.
                - Ppt. decomposed with $Ba(OH)_2$
                  - Evaporated to dryness and extracted with absolute alcohol
                    - Precipitated with $PtCl_4$
                      - Filtrate
                        - Fraction S111 {<200 r. 1000 b.
                      - Ppt. extracted with H_2O
                        - Soluble decomposed with H_2S
                          - Fraction S110 {<500 r. <200 b.
                        - Insoluble decomposed with H_2S + HCl
                          - Fraction S114 {1000 r. >1000 b.
                    - Insoluble Residue S107 {<100 r. b.
                  - Residue after digesting with dilute H_2SO_4 S106 {<2000 r. b.
        - Precipitated to pH 8.0
          - Fraction S116 {<100 r. 1000 b.
        - Filtrate S105 {15,000 r. 3000 b.

* r. indicates estimated doses for rats; b., for birds.

half of the precipitate was soluble even in copious amounts of water. To our surprize the water-insoluble portion yielded upon treatment with hydrogen sulfide much more activity than the water-soluble portion.

The above results show a wide scattering of activity in many fractions, which is in great contrast to Jansen and Donath's reported results with rice polish. The aggregate losses were so great that it seemed wholly useless to attempt the final steps of fractionation with absolute alcohol and acetone. We are unable to say at what point the greatest loss of activity occurred as, in order to conserve material, we did not test the "active" fraction at each successive stage. The discards account for substantial losses but what happened to the major part of the original activity remains unknown. Destruction in process seems the most plausible explanation. On the whole the results with yeast offered only vague confirmation of Jansen and Donath's claims.

Mention should be made of the fact that several fractions, Fractions S108, S112, and S111, showed much more activity on pigeons than on rats, suggesting the possibility that the "third factor" was present in them. The final fraction, as well as several discards, showed the property of protecting pigeons against polyneuritis without causing substantial increase of weight from previously attained subnormal levels.

Rice Polish Experiments.

A fractionation of 126 kilos of rice polish was carried out strictly according to Jansen and Donath with three minor exceptions as noted below. One of these was the substitution of methyl alcohol for ethyl alcohol in the extraction of the polish; another was the separation of the water-soluble platinichloride precipitate from the insoluble, as was done in the above fractionation of yeast; and third we did not decolorize with norit at any point.

In two respects we departed from the procedure as outlined in the yeast experiments. We precipitated with phosphotungstic acid before the silver nitrate and baryta fractionation, a procedure sanctioned by Jansen and Donath. In the rice polish experiment we also omitted the solution and reprecipitation of each of the silver nitrate fractions, as a rereading of the Jansen and Donath paper indicated we should have done in the case of yeast. How-

ever, the fraction from pH 6.5 to 8.0 was dissolved and reprecipitated as prescribed.

The activity was followed throughout the process by experiments on both rats and pigeons. The rat experiments in this case involved no use of autoclaved yeast, which we had in the meantime found to be liable to contamination with antineuritic vitamin (4). Consequently in these experiments a dose for rats is the daily amount necessary to protect against polyneuritic symptoms on the Sherman and Spohn Diet 107 without regard to weight changes.

The weights of rats under these conditions, with a supplement of no other vitamin B factor than the antineuritic, are represented by substantially horizontal lines. Occasional cases of pellagra-like symptoms are noted, including sore eyes and nose and dermatitis of the fore paws, but these symptoms do not appear early or with great regularity, and death does not ensue for several weeks, never within 60 days after the vitamin B-free diet is begun at the age of 4 weeks. The daily dose required for protection against polyneuritis under these conditions is not far from that required to produce normal growth when autoclaved yeast is added as a further vitamin B supplement. Three rats were used in each test of each fraction noted. When the trial dose proved too small as indicated by the early occurrence of polyneuritic symptoms or death we continued the experiment with the remaining animals to the end of the 60 day period and started a fresh group of rats on another trial dose, usually twice as large as the first trial dose. If the first trial dose proved ample, smaller trial doses were administered to other groups of rats till one was encountered which permitted the appearance of polyneuritis. Two trial doses were often administered to two groups simultaneously. The dose actually required was estimated by interpolation. In some cases polyneuritis developed very late in the test, which was regarded as evidence that the trial dose was nearly sufficient and the dose required was estimated at 1.5 to 2.0 times the trial dose. It was usually possible to forecast roughly what activity a given fraction was likely to possess. Control rats without antineuritic supplement regularly died within 35 days after a period of decline of 5 to 15 days. Twenty-nine groups of three rats each were required for the evaluation of the eighteen fractions from rice polish which were tested by this method.

We are far from satisfied with the quantitative aspects of this method of testing for antineuritic vitamin and accordingly also tested certain fractions by the use of the same diet with an additional supplement of autoclaved yeast. No serious discrepancy was encountered. Some of these results will be discussed later.

Our pigeon testing method in the rice polish experiments involved preliminary depletion by feeding on white rice alone with subsequent dosing on alternate days to determine the minimum quantity required to protect against polyneuritis regardless of the weight curve. We were led to adopt this modification as we found that the more highly refined fractions in the series of steps had very little influence on the weight of birds when used as the sole addendum to a polished rice diet. Yet certain of these fractions protected very effectively against polyneuritis. However it occasionally happened that one bird of the group of four used for testing each fraction developed polyneuritis, while the remainder did not, in which case the behavior of the apparently exceptional bird was discounted in making an estimate of the dose required. In one extreme case one bird failed to contract polyneuritis on a dose which proved to be only one-fourth that required to protect another bird in the same group. In order to economize animals we often changed the trial dose in our pigeon experiments after a period of 2 to 3 weeks, doubling or halving the dose as the results suggested. When we found we had made a very bad estimate in the first trial dose we began again with a fresh group of birds. The duration of the pigeon experiments was usually about 2 months; a few involving fractions of very low activity terminated with death of the animals in 4 to 6 weeks; several extended for 4 months. Forty-two groups of four birds each were used in the pigeon tests. All birds were weighed and dosed every other day.

The accuracy of the estimates of the minimum required dose is, of course, not high. It was especially difficult to compare the activity of the early crude fractions with the later more refined ones, as the differences appear to be qualitative in part. However, it may be safely said that in no case is the dose which we have set down as required so low as to fail to retard the development of polyneuritis by at least 3 weeks, nor so high that it could be halved without the development of polyneuritis within that time. We are by no means satisfied with either the rat or pigeon

method of test. A diet is needed for each species which will permit normal nutrition with a vitamin B_1 supplement and which will kill in a few weeks without it. We are endeavoring to secure a physiologically pure supply of the "third factor," vitamin B_3, required by pigeons and a form of autoclaved yeast or its equivalent which is devoid of vitamin B_1 for use with rats. Until these are obtained the testing for vitamin B_1 will be unsatisfactory.

The early steps of the rice polish fractionation (Diagram 2) are remarkably efficient, very little activity being lost in the extracted polish, or in the filtrate from the acid clay. The clay, after extraction with baryta, retained only a small amount of activity. In order to economize material we did not test the baryta extract or any of the presumably active fractions until we reached the absolute alcohol extraction stage. By this time only 25,000 bird doses were left, or about 10 per cent of the activity in the original rice polish. The largest single loss accounted for in any of the discards was in the silver nitrate precipitate up to pH 4.5 (Solution 7), which contained nearly one-fifth of the original activity. Small losses occurred at many other points but the greater part of the losses cannot be accounted for in the discards. Again as in the yeast experiment destruction in process seems the most plausible explanation. The most probable destructive operations are those involving alkalinity. However, all operations were carried out with care and expedition and the results of our feeding tests appear to be fairly harmonious and reproducible. Lack of experience with Jansen and Donath's rice birds forbids a comparison, but we wish to call attention to the much longer duration of our own feeding experiments. We are forced to the conclusion, tentatively at least, that Jansen and Donath underestimated their losses by destruction and in discards prior to the alcohol-acetone fractionation with which their process concludes. However, at every stage of fractionation we found that the activity followed the course indicated by Jansen and Donath rather than the opposite one. There is every indication that we are dealing with the same substance by a process which is qualitatively reproducible up to this point.

We endeavored to further fractionate (Diagram 3) the discard Solution 7 which represented the largest single traceable loss. This solution was brought to dryness, taken up in absolute alcohol,

DIAGRAM 2.

Rice polish {180,000 r.* / 250,000 b.} ——— Extracted polish {40,000 r. / 20,000 b.}

Extract (234 liters)

Active clay {150,000 r. / 240,000 b.} ——— Filtrate <10,000 b.

Treated with baryta

——— Residue 2 {4000 r. / 1000 b.}

Solution treated with phosphotungstic acid

——— Filtrate 3 {<1000 r. / <1000 b.}

Precipitate fractionated with acetone

——— Acetone-insoluble Fraction 4 {2000 r. / <1000 b.}

——— Filtrate 8 {<2500 r. / <2500 b.}

Reprecipitated phosphotungstates decomposed with baryta, and fractioned with $AgNO_3$ and baryta

Precipitated to pH 4.5. ——— Filtrate 6 {<1000 r. / <2000 b.}

Freed of Ag, Solution 7 {13,000 r. / 50,000 b.}

Precipitated to pH 6.5, decomposed with HCl, desiccated and extracted with absolute alcohol

Solution 9 25,000 b. ——— Insoluble Residue 10 {2500 r. / <5000 b.}

Treated with $PtCl_4$

——— Filtrate 14 1.60 gm. solids {None r. / <2500 b.}

Ppt. extracted with water

——— Extract freed of Pt = Solution 11 {7000 r. / 12,000 b.}

Residue extracted with dilute HCl

——— Extract freed of Pt = Solution 12 {5000 r. / 8000 b.}

Residue digested with HCl + H_2S

——— Filtrate 13 {Negligible r. / " b.}

* = indicates estimated doses for rats; b. for birds

leaving an insoluble residue. The absolute alcohol solution was precipitated with platinic chloride. The filtrate and precipitate were separated and each was freed of platinum with hydrogen sulfide. The three fractions so produced were tested physiologically. The results furnished further verification of the deportment of the vitamin hydrochloride toward absolute alcohol and toward platinic chloride in absolute alcoholic solution, as claimed by Jansen and Donath and verified by ourselves in the fractionation of the main portion. It also served to confirm the presence in Solution 7 of a substantial amount of vitamin, though less than was indicated by the tests on the original Solution 7. This is the most serious discrepancy in the results of our feeding tests.

DIAGRAM 3.

$\frac{4}{5}$ Solution 7

Desiccated and extracted with absolute alcohol

Extract——————————————Residue 15 $\begin{cases} 6000 \text{ r.*} \\ 5000 \text{ b.} \end{cases}$

Precipitated with $PtCl_4$

——Ppt. decomposed with $HCl + H_2S$ = Solution 16 $\begin{cases} 12{,}000 \text{ r} \\ 12{,}000 \text{ b} \end{cases}$

Filtrate freed of Pt = Solution 17 1.79 gm. solids $\begin{cases} \text{None b.} \\ \text{" r.} \end{cases}$

* r. indicates estimated doses for rats; b., for birds.

Comparison with our previous fractionation of yeast brings out a considerable discrepancy in the solubility in water of the active platinichloride precipitate. In our yeast experiments the active substance did not dissolve in water; in the rice polish experiments it did so to a very substantial degree but not completely.

A further comparison of the two series of experiments with respect to the occurrence of indications of the "third factor" seems justified. We have already noted that in the yeast fractionation certain fractions were found which were active for pigeons but not for rats. We found no such fractions in the rice polish

experiments, perhaps partly because our testing methods were modified so as to betray such a fact less readily. It is also probably a significant fact that the duration of the baryta treatment of the fullers' earth was shorter in the first series of experiments than the corresponding treatment of the acid clay when dealing with rice polish. Destruction of the "third factor" is known to occur rather rapidly in alkaline solution.

We may now turn our attention to the final purification with alcohol and acetone. This process, from visual indications, proceeded very much as we were led to expect from Jansen and Donath's description, and we were very hopeful of being able to

TABLE I.

Four-Fifths of Solution 11 Fractionated with Alcohol and Acetone According to Jansen and Donath Yielded These Results.

Fraction.	Description.	Total weight.	No. of bird doses in fraction.	Weight of daily dose.
		gm.		*mg.*
D	Earliest discards insoluble in alcohol.	0.128	600	0.20
E	Later discards insoluble in alcohol.	0.132	600	0.20
F	Earliest discards not precipitated by acetone.	0.182	1800	0.10
G*	Next " " " " "	0.463	3800	0.12
H	" " " " " "	0.157	600	0.25
C	Last " " " " "	0.129	500	0.25
A*	First crop of crystals.	0.032	500	0.06
B	Second " " "	0.003	<10	

* Also tested with rats, G = 3800 doses; A = 150 doses.

duplicate their results. We did not succeed in doing so, however, as at no stage of our procedure did we observe any acicular crystals, nor indeed any crystals of non-hygroscopic character. The two crops of crystals finally obtained were blocky tablets and gave no evidence of approaching purity. Since the quantity remaining was now so reduced that both analysis and physiological tests were impossible, we stopped our efforts to purify the material further. We made certain combinations of our discards as indicated in Table I, and physiologically tested each fraction with birds, and in the case of two fractions, with rats as well. The results showed a wide scattering of the activity. However, the

coarse crystals, Fraction A, representing our closest approach to Jansen and Donath's crystals in point of solubilities, were somewhat the most active weight for weight of all the fractions. They were about one-third as active as the crystals of the Dutch experimenters were reported to be.

We regard this as a partial confirmation of their results. That the confirmation is not more complete is perhaps not highly significant because our product resulting from the decomposition of water-soluble platinichloride was presumably not as pure as the product with which Jansen and Donath began their alcohol-

DIAGRAM 4.

$\frac{4}{5}$ Solution 12, desiccated, dissolved in water and precipitated with picric aci

——Ppt. freed of picric acid = Solution 18 500 b

Filtrate freed of picric acid with $ChCl_3$ and treated with picrolonic acid

——Filtrate freed of picrolonic acid = Solution 19 2400 b.

Ppt. freed of picrolonic acid with ether and treated with $HgCl_2$

——Precipitate freed of Hg with H_2S = Solution 20 150 b.

Filtrate freed of Hg, dried, and treated with $AuCl_3$

——Precipitate freed of Au = Solution 21 500 b.

——Filtrate " " " = " 22 <200 b.

* b. indicates estimated doses for birds.

acetone purification. Our Solution 11 contained only 5 per cent of the activity of the original rice polish, while Jansen believed that 25 per cent of the original activity was present in their case at this stage. The gross weights of the two fractions were very similar. It is obvious that it might be impossible to recover any pure substance from a cruder source by a process adapted to recover only a small fraction of the total present. Moreover, we have since learned by conversation with Dr. Jansen that we used acetone in smaller proportions relative to alcohol present than was done in the original work, and that our fractionation with these solvents was less elaborate.

Following the statements of Jansen and Donath regarding precipitation reactions of the vitamin, we attempted to obtain some purer substance from Solution 12 by first clearing with picric acid; precipitating the active substance with picrolonic acid; decomposing the picrolonate, and clearing with mercuric chloride, and finally

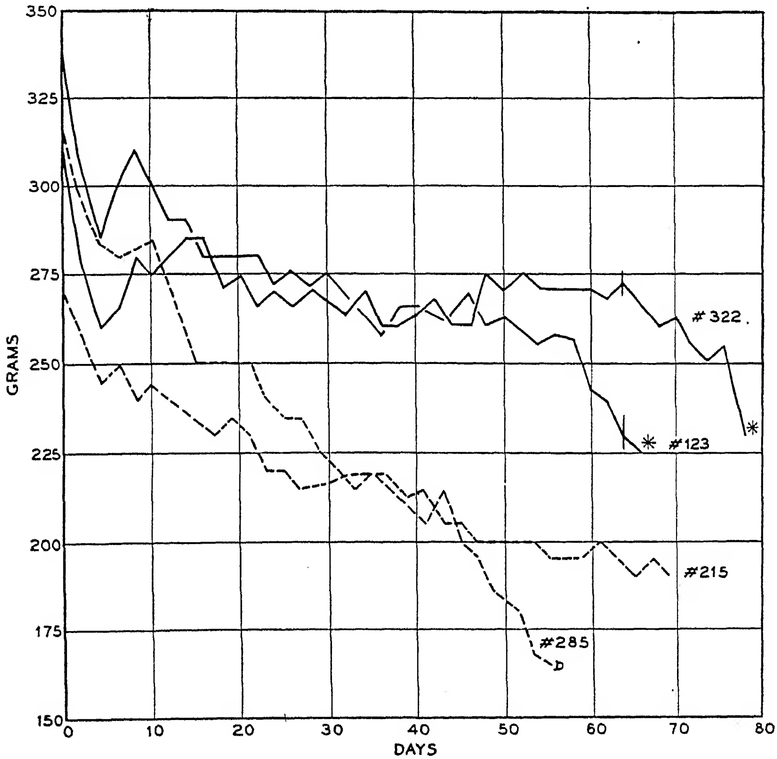

Chart I. Pigeons on polished rice. Pigeons 322 and 123 received 0.3 mg. of Solution 16 every other day till the 64th day. Pigeons 215 and 285 received 0.05 mg. of Solution 21 every other day after the 21st day.
* = polyneuritis. D = death.

precipitating with gold chloride as shown in Diagram 4. Each of the products was tested on birds. The results indicate that only one of the fractions is more active weight for weight than the coarse crystalline Fraction A, referred to above. This fraction, Fraction 21, obtained from the gold precipitate seems at least half

as active as Jansen and Donath's final product. (See Chart I.) In general this affords a considerable measure of additional confirmation of Jansen and Donath's report, particularly with reference to the precipitation reactions of the active substance.

The weight of substance per dose for rats and for pigeons for a number of our products is given in Table II. Up to this point we may summarize our findings with rice polish by stating that there is a good qualitative correspondence with Jansen and Donath but a poor quantitative one.

A further opportunity to test the significance of our respective findings was offered by the kindness of Dr. Jansen who supplied us

TABLE II.

Weight and Activity of Several Fractions.

Fraction No.	Approximate weight of fraction.	Weight of daily dose.	
		Rats.	Pigeons.
	gm.	*mg.*	*mg.*
11	1.6200	0.23	0.13
12	0.6000	0.12	0.08
14	1.6000		>0.70
16	1.9000	0.15	0.15
17	1.7900	>1.00	>0.70
18	0.0492		0.10
19	0.1200		0.05
20	0.0080		0.10
21	0.0130		0.04
22	0.0130		>0.06

with 5.6 mg. of his acicular crystals of the vitamin hydrochloride. Dr. Jansen demonstrated in the presence of one of us that a water solution of these crystals gave the typical and beautifully crystalline picrolonate and gold double salt described in the original work. A trial of the melting point of the hydrochloride crystals, however, betrayed to Dr. Jansen the fact that they were not quite pure though very well formed. Dr. Jansen estimated from the degree of purification to which they had been subjected that they were at least 95 per cent pure. It seems well worth while to report in detail the results of our pigeon and rat tests of these crystals (Charts II and III). For comparison, similar data are given in Chart I with respect to some of the more advanced products of our

own fractionation, *viz.* Solutions 16 and 21. The Jansen crystals were tested in two ways. In one case the birds were first fed

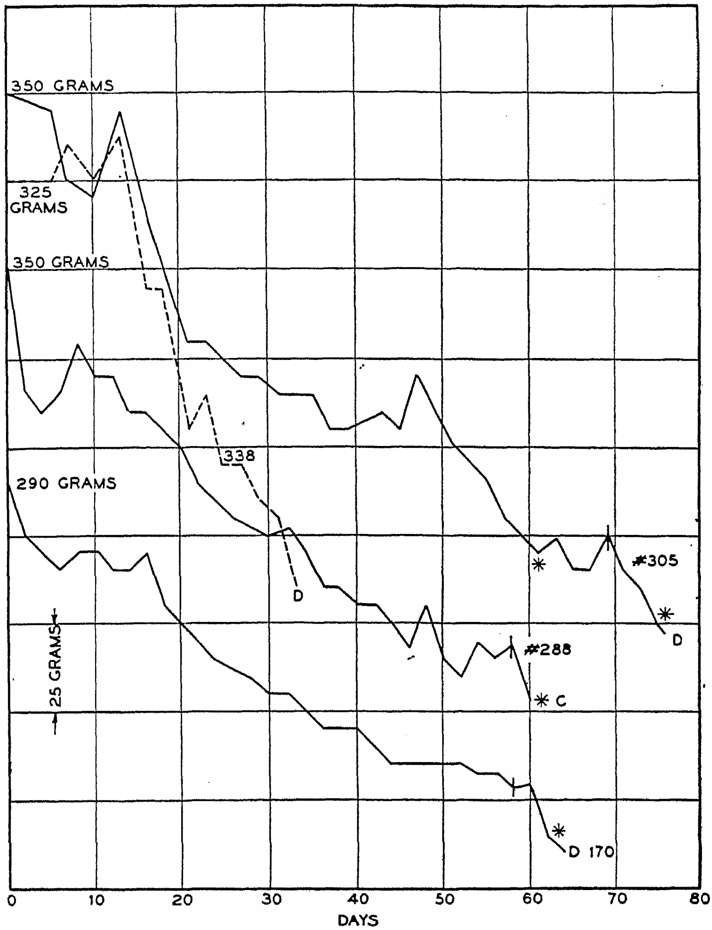

CHART II. Pigeons on polished rice. Pigeon 305 received 0.04 mg. of crystals from the 21st to 61st day, and 0.08 mg. of crystals from the 61st to 69th day. Pigeon 338 received 0.04 mg. of crystals from the 21st day till death. Pigeon 288 received 0.04 mg. of crystals from the 1st to 50th day, and 0.08 mg. of crystals from the 50th to 58th day. Pigeon 170 received 0.04 mg. of crystals from the 1st to 58th day.

* = polyneuritis. C = cured. D = death.

polished rice alone until considerably depleted of vitamin and then given doses on alternate days of the solution under test; in the other case the dosing on alternate days was begun as soon as the birds were put on the polished rice diet. The former represents our usual method of test; the latter resembles that used by Jansen

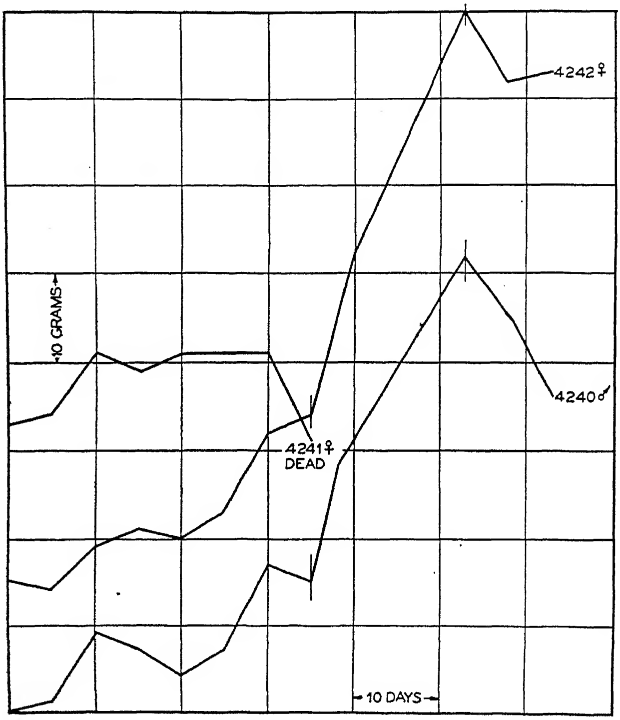

CHART III. Rats on the Sherman and Spohn diet. All received 0.5 gm. of neutral autoclaved yeast from the 15th day onward. Rats 4240 and 4242 received 0.04 mg. of crystals daily from the 35th to 53rd day.

and Donath when dealing with pigeons. The doses were given by mouth with a pipette in both cases. We used no supplements of cod liver oil or meat powder as we should thereby have lost comparability to our own previous experiments, many hundreds in number. Consequently our experiments with Dr. Jansen's crys-

tals are not strictly comparable with the reported pigeon experiments of the Dutch workers.

Of the two pigeons which were first depleted of vitamin by feeding for 21 days on polished rice alone, Pigeon 338 died on the 33rd day, while receiving every other day 0.04 mg. of the crystals in solution. No distinctive symptoms of polyneuritis were noted before death. The other bird, Pigeon 305, continued on the same dosage for 25 days longer before any evidence of polyneuritis was noted. At this time the bird had a staggering gait and could not fly to its perch. The daily dose was doubled on the 61st day and the next day the bird was on its perch. Improvement continued for 8 days, when the dosing was discontinued and the bird contracted polyneuritis of definite type 7 days later.

Of the two birds, maintained on a white rice diet and dosed every other day with 0.04 mg. from the outset, one, Pigeon 288, developed polyneuritis (convulsions with head drawn forward) on the 50th day; thereafter it received a doubled dose under which treatment it improved and lost all distinct traces of polyneuritis. On the 58th day dosing was stopped and polyneuritis developed on the 60th day. It was cured with activated fullers' earth. The other bird, Pigeon 170, showed no evidence of polyneuritis on the smaller dose for 58 days. 6 days after the dosing was discontinued the bird died with evidence of polyneuritis.

As will be seen from the weight curves chart, all birds declined heavily in weight. Material of our own preparation, Solution 21, acts in the same way, and we believe that it will be found impossible to keep pigeons alive indefinitely upon a white rice diet supplemented by antineuritic vitamin alone. There does not seem to be much room for doubt that these crystals have great protective power against polyneuritis and that they contain the antineuritic vitamin. A dose of 0.04 mg. every other day appears to be somewhat too small.

To confirm these findings on pigeons we tested the crystals on three rats 28 days old, which were fed the Sherman and Spohn Diet 107, supplemented after 15 days by neutral autoclaved yeast. When polyneuritis developed in one rat we began dosing each of them daily with 0.04 mg. of the crystals in solution. The polyneuritic rat was in extremis and died in a few hours after the first dosing. The other two promptly and markedly increased in

weight for 17 days, when the dosing had to be discontinued for lack of material. They then promptly declined in weight (Chart III).

A fraction of a mg. of the crystalline material was sent to Dr. Maurice I. Smith of the Hygienic Laboratory for curative tests on rats. He has kindly reported favorable results to us. Dr. Roger J. Williams of the University of Oregon was also furnished 0.1 mg. and has reported elsewhere marked stimulation of yeast growth with the substance.

Of the purity of Jansen and Donath's product we can of course say little, much less of the correspondence of its composition to the formula $C_6H_{10}N_2O$. We are not fully convinced that the activity of these crystals cannot be due to an adhering impurity but have encountered no specific evidence in favor of such a view. Our own most potent product, Solution 21, has the same physiological properties and is effective in similar doses on both rats and pigeons. We are disposed to take Jansen and Donath's claims of isolation seriously.

We attempted, by combining Solutions 21 and 22, to detect a supplementary relationship between the two, such as was remarked by Guha and Drummond (5) with similar preparations. Our results were negative on pigeons. Lack of material prevented similar rat tests.

Attention is called to the evident qualitative difference between Solution 16 and Solution 21 as reflected by the better weight maintenance obtained with the former. We attribute this to the continued presence even at relatively advanced stages of fractionation of some factor other than the antineuritic vitamin. It might be objected that these two experiments are not strictly comparable since Solution 16 was given from the outset while Solution 21 was not administered during the first 3 weeks. However, this is not the explanation of the disparity referred to, for birds in another experiment, which received Solution 16 only, after a preliminary depletion period lost weight no more rapidly than the birds shown in Chart I. Also it is evident from Chart II that it made no sensible difference in the weight loss occurring on administration of Jansen and Donath's crystals, whether the dosing was begun early or late.

We regard as the most important result of the work described in

this paper, the evidence adduced that the pure antineuritic vitamin in small doses at least has scarcely any appreciable effect upon the the weight curves of pigeons on a polished rice diet. In the curves shown in Chart II, delay in onset of polyneuritis and death is the only evidence that we are dealing with the antineuritic vitamin. Actually we still entertained some doubt on this point till we had performed the experiments with rats, shown in Chart III.

Most of the work done in the past looking to the chemical isolation of the antineuritic vitamin has been done with pigeons and has involved considerable reliance on the weight curves. A notable exception is that of Peters and his associates (6) who for years past have preferred to measure activity by length of delay of recurrence of symptoms after administration of a curative dose. Peters' position seems in part at least to be vindicated by our results. Incidentally we have tested products prepared by Peters' methods with results which further justify our conclusions about the physiological properties of the antineuritic vitamin. To what extent reliance on weight curves has affected earlier conclusions can be estimated only very roughly, but it is not too much to say that some experiments must have been adjudged failures because they were chemically successful; *i.e.*, actually eliminated some impurity of favorable physiological effect but foreign to the antineuritic vitamin itself. The actual non-response of the weight curve to the antineuritic vitamin has also great bearing on all the work which aimed at an improved synthetic diet for pigeons (7) and upon the matter of vitamin B_3 (2). The complication of forced feeding *versus* feeding *ad libitum* is a serious bar to reinterpretation of these results. It also raises a question as to whether various substances of synthetic or natural origin which have been tested from time to time in the past may not have been seriously misjudged. We can no longer entertain any doubt that pigeons on polished rice are suffering from a multiple deficiency of vitamins and cannot presume without further evidence that human beriberi does not also involve multiple deficiencies (8) which may account for its variant symptomatology.

SUMMARY.

1. Jansen and Donath's process (with minor modifications) applied to brewers' yeast leads to heavy losses of activity and a

negligible recovery of active material in the selected fraction after platinum chloride precipitation.

2. The same process (with slight variations) applied to rice polish leads to smaller losses and better recovery but only one-fifth as much activity was present at the platinum chloride stage as reported by Jansen and Donath.

3. Further fractionation with alcohol and acetone did not yield crystals as described by Jansen and Donath, but the relative distribution of activity in various fractions corresponded roughly with their report.

4. Fractionation of a by-product with reagents mentioned by Jansen and Donath betrayed no error in their account of the precipitation reactions of the vitamin, and yielded a non-crystalline material of substantially the same physiological properties as the Jansen and Donath vitamin.

5. Vitamin hydrochloride crystals kindly supplied by Jansen and Donath were tested on pigeons on a polished rice diet. In doses of 0.04 mg. daily, polyneuritis and death were much retarded but weight loss was not avoided.

6. Rats on a synthetic diet including autoclaved yeast showed a marked growth response to daily doses of 0.04 mg. of the same crystals.

7. Our own more advanced fractions also react qualitatively and approximately quantitatively in the same fashion as Jansen and Donath's crystals upon both rats and pigeons.

8. We conclude that Jansen and Donath's crystals (whether pure or not we cannot say) possess antineuritic properties.

9. The antineuritic vitamin in small doses does not affect the weight curves of pigeons on polished rice.

10. Birds on this diet suffer from a multiple deficiency.

We gratefully acknowledge generous financial support of this work by the Carnegie Corporation through the intermediary of the Carnegie Institution of Washington. Our thanks are also due to Dr. Walter H. Eddy for advice and assistance and to the Misses Minerva Kellogg and Marion Ammerman, and Mr. John C. Keresztesy for assistance in the care of animals and in preparing experimental material.

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THE EFFECT OF ANTINEURITIC VITAMIN PREPARATIONS ON THE GROWTH OF YEASTS.

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In the first study of yeast growth carried out by the senior author (1) the conclusion was drawn that yeast needs for its normal nutrition an unknown substance (or group of substances) which was thought to be identical to what was then frequently called water-soluble B. This study was concerned with the strain of yeast commonly employed at that time in the manufacture of bakers' yeast and which has been designated as "old process" (O. P.) yeast.

It is now clear that vitamin B possesses a multiple nature and that yeast growth stimulants are of diverse character depending on the type of yeast used. While the conclusion as to the identity of the yeast growth stimulant and vitamin B is plainly unjustified, the possibility remains that certain components of vitamin B may act as yeast growth stimulants. The work here presented indicates that this is actually the case.

It is clear from the results published in a previous paper (2) that different strains of yeast show at least two types of behavior toward nutrilites. Old Process Bakers' Yeast and Yeast 578 of the American Type Culture Collection, both clearly require at least two separable nutrilites for normal growth, one of which is adsorbed by Lloyd's reagent, fullers' earth, etc., and the other which is not adsorbed. Gebrüde Mayer yeast on the other hand seems to be stimulated very readily by material which has failed to be adsorbed by fullers' earth.

In the original work of Wildiers (3) which led to the discovery of "bios," it seems reasonably certain that a yeast of the type represented by Gebrüde Mayer was used. This conclusion is based on the fact that Wildiers found "bios" not to be precipitated by the ordinary precipitants including phosphotungstic acid.

Recent work in this laboratory (4) has shown that the yeast growth stimulant effective for Gebrüde Mayer yeast follows Wildiers' description. On the other hand, various precipitants including phosphotungstic acid render extracts ineffective for O. P. yeast or Yeast 578, by removing an essential factor.

Since the antineuritic component of vitamin B is readily adsorbed by fullers' earth (also precipitated by phosphotungstic acid), it is clear that this component can materially affect only yeasts of the type represented by O. P. yeast and Yeast 578. Our study, outlined below, of the effect of antineuritic vitamin preparations has accordingly been carried out principally on Yeast 578 and to some extent on O. P. yeast.

Determination of Potency as Applied to the Adsorbed Factor.

Several yeasts require for rapid growth two distinct complementary factors one of which is readily adsorbed under proper conditions, by fullers' earth, or Lloyd's reagent. In less extensive experiments silica gel and activated charcoal have been found to adsorb this active material. The other factor is not readily adsorbed by these or any other adsorbents tried. Since we have been concerned with the concentration and testing the potency of preparations containing the adsorbed factor, we have introduced into the control culture medium a fixed quantity of the unadsorbed residue which was described in a previous publication (2) without which the adsorbed factor is ineffective.

We have found through experience that it is not possible to judge with sufficient accuracy the potency of preparations, so far as the adsorbed factor is concerned, merely by determining the yeast growth produced under controlled conditions. It is much more satisfactory to use a particular preparation as a standard in each test and compare side by side the effects of preparations of unknown strength to the effects produced by a known amount of standard material. For the standard we have chosen a particular batch of "fullers' earth solids," that is the solid material extracted with baryta from fullers' earth which had been activated by shaking with a rice polish extract. 1 gm. of this material contains practically all of the adsorbed factor from 300 gm. of moderately active rice polish. We shall designate this standard material as

"S" for the sake of brevity and assign it arbitrarily a potency factor of unity.

In each test in which the potency of a preparation is determined cultures containing the following ingredients are made up as a matter of routine.

1. 10 cc. synthetic medium + 1 cc. H_2O + 1 cc. yeast suspension.*
2. 10 cc. synthetic medium to which 16 mg. "unadsorbed residue" is added (control medium) + 1 cc. H_2O + 1 cc. yeast suspension.
3. 10 cc. control medium + 1 cc. solution containing 0.8 mg. "S" + 1 cc. yeast suspension.
4. 10 cc. control medium + same containing 0.4 mg. "S" + 1 cc. yeast suspension.
5. 10 cc. control medium + same containing 0.2 mg. "S" + 1 cc. yeast suspension.
6. 10 cc. control medium + same containing 0.1 mg. "S" + 1 cc. yeast suspension.

* The yeast suspension contains 640,000 cells per cc. and the seeding, etc., is carried out in accordance with previous publications (2). The initial concentration of yeast cells in the culture is about 53,000 cells per cc.

The material to be tested is introduced in different dosages (two or more) into separate portions of control medium and seeded in the same way and at the same time with yeast.

At the end of 18 hours of undisturbed growth at 30° the cultures are dosed with 0.5 cc. of formalin and shaken mechanically for 10 minutes. The relative amounts of yeast in the suspensions are then determined by the method involving the use of a sensitive thermocouple (5).

For the present purpose the actual quantities of yeast need not be calculated, but the galvanometer readings can be used directly. The galvanometer readings for the four cultures containing varying quantities of "S," as well as that in which the control medium alone is used, are plotted. Fig. 1 shows a typical curve obtained in this way (Yeast 578). Curves obtained on different occasions differ somewhat in the magnitude of the values due to unavoidable differences in seeding etc., but the shapes of the curves are all similar. The dosages of the unknown preparations are estimated so that the growths produced will be comparable to those resulting in the standard series mentioned above. Ordinarily two dosages are used, one twice that of the other. The galvanometer deflec-

tion produced by an unknown is read off the curve in terms of the amount of "S" to which the unknown is equivalent. By dividing the actual dosage of the unknown into the value for the equivalent amount of "S" the potency factor of the unknown is obtained. Thus if 0.0025 mg. of an unknown run alongside the series plotted in Fig. 1 produces a galvanometer deflection of 8.0 it is equivalent to 0.3 mg. of "S." Since "S" is arbitrarily assigned a potency factor of unity the unknown has a potency fac-

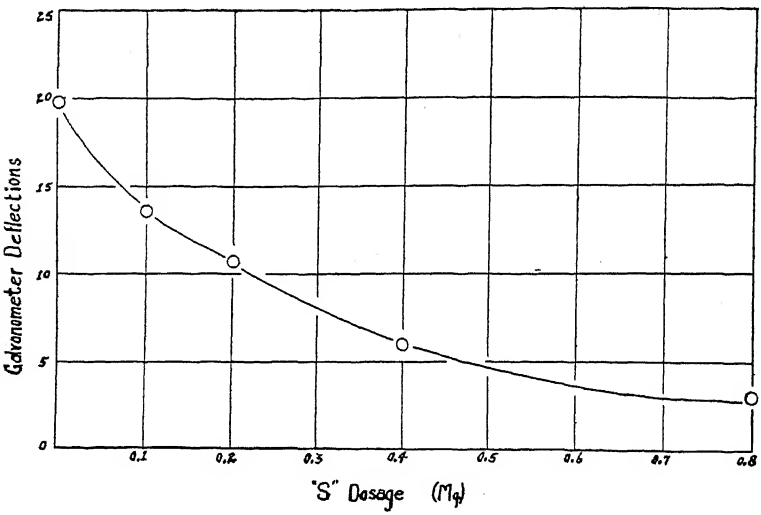

FIG. 1. Curve used in potency determinations.

tor of $\frac{0.30}{0.0025}$ or 120. This illustrates the method of calculating the potency factors listed below.

Use of the Jansen-Donath Procedure for Concentrating the Adsorbed Factor.

In a previous paper (2) was outlined briefly the method of preparation of a concentrate designated as "Z_2." The later stages in its preparation (from yeast extract) were based upon the procedure of Jansen and Donath (6) for purifying the antineuritic vitamin. For purposes of comparison it should be stated that

"Z_2" has a potency factor of 68. Triplicate determinations with dosages of 0.0005 mg., 0.0025 mg., and 0.00125 mg. (in 12 cc.) gave the values 69, 66, and 68 respectively. These values show a closer agreement than can ordinarily be expected.

Partly for the sake of following Jansen and Donath's work more closely and partly for reasons of economy, our later work in concentrating the adsorbed factor has been carried out with rice polish and rice bran as the raw materials. Certain grades of rice bran we find much richer than rice polish which represents the last portion of the grain removed in the milling process.

Without going into great detail it may be stated that the various procedures used by Jansen and Donath with the exception of the final ones, seem to be effective in concentrating the adsorbed factor under investigation. These procedures include (1) adsorption on fullers' earth or acid clay, (2) removal with baryta, (3) precipitation with phosphotungstic acid, and decomposition of the phosphotungstates with baryta, (4) fractionation with silver nitrate and baryta at different pH values, (5) decomposition of the silver precipitates with hydrochloric acid, (6) solution of the material in absolute alcohol, (7) precipitation with chloroplatinic acid, (8) decomposition of the chloroplatinates with hydrogen sulfide.

After precipitating with phosphotungstic acid and decomposing the phosphotungstates the potency factor of the material so obtained was 6 to 8. The material precipitated by silver nitrate from pH 4.5 to 6.8 was most active (in accordance with Jansen and Donath's work) and had a potency on the average of about 20. The material obtained by platinum precipitation had a potency factor of 30 to 40. Later, with extracts from rice bran as a starting material, all of the values mentioned above were higher so that a decomposed platinum precipitate was obtained which possessed a potency factor of about 80. When such a material was dissolved in absolute alcohol and absolute acetone was added, a precipitate formed which, when filtered off, left soluble material with a potency factor of about 120. This represents the most potent material we have so far separated from rice bran.

In the process of concentration there were inevitably rather large losses of activity. These were encountered especially in the silver fractionation, to a considerable extent in the platinum precipita-

tion, but not in the process of fullers' earth adsorption and removal nor in the phosphotungstic acid precipitation and the subsequent decomposition with baryta. Since these results were in line with the experience of Jansen and Donath they seemed to justify the hypothesis that the adsorbed factor and the antineuritic vitamin are closely related.

When the method of Jansen and Donath was followed beyond the chloroplatinic acid precipitation, the adsorbed factor was not effectively concentrated. The addition of acetone to a solution of the material in absolute alcohol failed to yield a more active precipitate (Jansen and Donath) but instead the soluble material was more active. In one experiment the potency factor of the precipitate was 24 while that of the soluble material was about 120.

Tests on the Preparations of Williams and Waterman.

Through the kindness of R. R. Williams and R. E. Waterman we were furnished with samples of their preparations obtained in an attempt to duplicate the work of Jansen and Donath on the antineuritic vitamin. They had a different source of raw material, used a superior type of adsorbent clay (the same as used by Jansen and Donath), and worked upon a larger scale; their preparations which can be identified by reference to their paper (7), have higher potency factors than corresponding preparations made in this laboratory. The potency factors of the various preparations furnished to us are listed in Table I with the corresponding purity factors determined by their animal tests.

The purity factors are based on the assumption that 0.02 mg. of pure antineuritic vitamin are required daily by the pigeon and 0.03 mg. by the rat.

It is to be noted that Samples 11 to 17 inclusive, which represent fractions obtained prior to the alcohol-acetone fractionation stage show an excellent agreement in relative rating by both yeast tests and animal tests. It will be recalled that yeast tests on our own preparations at corresponding stages were in agreement with the assumption that the antineuritic vitamin is identical with the adsorbed factor of yeast stimulatory effect. Beyond this stage marked disagreement with such an assumption appeared both in the results of Williams and Waterman and those of our own laboratory. In both laboratories the fraction not precipitated by acetone was

TABLE I.

Potency and Purity Factors of Various Preparations of Williams and Waterman.

Sample No.	Potency factor.	Purity factor.	
		Pigeons.	Rats.
11	128	15	15
12	163	25	25
14	67	2	0
16	107	13	20
17	14	2	0
A	20	35	15
C	16	8	
D	61	10	
E	112	10	
F	228	20	
G	320	15	25
H	150	7	
18	45	20	
19	120	40	
20	48	20	
21	53	50	
22	3*	<35	

* Mold had grown on this preparation before it was submitted for the yeast test.

TABLE II.

Vitamin Crystals of Jansen and Donath.

Medium.	Galvanometer reading.	Potency factor.
Synthetic	35.3	
Control (containing 16 mg. U.R.*)	19.8	
" +0.8 mg. "S"	2.1	
" +0.4 " "	4.9	
" +0.2 " "	7.8	
" +0.1 " "	10.9	
" +0.004 " "C"	4.7	110
" +0.002 " "	7.5	146
" +0.001 " "	9.5	137
" +0.0005 " "	13.8	136

* U. R., "S," and "C" stand respectively for our unadsorbed residue, standard preparation, and the Jansen crystals.

much the more potent for yeast. It will be noted, however, that the preparation Sample G which is most effective for yeast still possesses a marked antineuritic activity especially for rats. In Samples 18 to 22 inclusive, which represent an attempt by Williams and Waterman to purify the vitamin by the use of precipitants, the correlation of yeast and animal tests is also poor.

Testing the Vitamin Crystals of Jansen and Donath.

We were fortunate in being able to obtain through the courtesy of B. C. P. Jansen and R. R. Williams a small sample of relatively pure crystals of the antineuritic vitamin as prepared by Dr. Jansen in Amsterdam. A 0.1 mg. sample dissolved in 1 cc. of dilute alcohol served for a number of tests. The results of the first test on this crystalline material are indicated below. The crystalline material is designated as "C" (Table II).

The potency of this crystalline vitamin preparation is thus about twice that of the "Z_2" referred to in a previous publication. If it had been tested earlier in our work before we had tested the more potent preparations of Williams and Waterman, we would have been even more greatly impressed with its high potency. The potency of the crystals is such that 1 part in 100,000,000 parts of the culture medium is easily detectable, in experiments in which the original yeast seeding is very small. (High grade conductivity water contains ionic impurities much in excess of this concentration.)

DISCUSSION.

Serious attempts to purify the adsorbed yeast nutrilite lead to the conclusion that the active principle is not a single substance. The high potency of the antineuritic vitamin prepared by Dr. Jansen indicates that it is one of the substances which is concerned in yeast growth stimulation. It is possible, of course, that the stimulation produced by these crystals may be due to adhering impurities. This seems very unlikely, however, because of the relatively high potency of the crystals as well as their comparative purity, and also because of their method of preparation. The material precipitated by acetone from absolute alcohol both in our experiments and those of Williams and Waterman is much less potent for yeast growth than the final material obtained by Jansen and Don-

ath by fractionating material of this same character. In the fractionation process they presumably removed material which is physiologically inactive both to yeast and to animals. Further evidence indicating the effectiveness of more than one substance in yeast growth stimulation is the existence of the very active "G" preparation and the fact that large losses of activity are entailed in the silver nitrate-baryta fractionation, and to some extent in the platinum precipitation. It seems likely that these losses are due to discarding some active substance other than the one which makes the precipitated material effective.

It is interesting and perhaps significant that as attempts to purify vitamin B progress, evidence also accumulates as to the increasing complexity of what was at one time thought to be a single substance.

The testing of preparations for their effect on yeast growth must be carried out with considerable care but nevertheless the time and material consumed in carrying out these tests is insignificant compared with that involved in animal tests. From one standpoint it would seem desirable to use yeast tests as a tool in the purification and isolation of vitamin fractions. There seems to be now a strong probability that a purification of all the substances in the adsorbed yeast nutrilite would mean a simultaneous purification of substances which are effective in animal nutrition.

We wish to acknowledge our indebtedness to Standard Brands, Incorporated, successor to the Fleischmann Company, for supporting the fellowship which made this work possible and to the Research Committee of the University of Oregon for generous grants of funds.

SUMMARY.

1. Certain yeasts do not require for growth stimulation any substance which is adsorbed readily by fullers' earth. Only those yeasts which require such material can be materially affected by antineuritic vitamin preparations.

2. A satisfactory method is described for determining quantitatively the potency of preparations with respect to their content of the readily adsorbed yeast nutrilite.

3. The "bios" of Wildiers is not precipitated by phosphotungstic

acid (or other common precipitants) nor presumably adsorbed by fullers' earth. It probably has no relationship to the antineuritic vitamin.

4. However, the method of Jansen and Donath for preparing the antineuritic vitamin is very effective for concentrating the adsorbed yeast nutrilite, as far as the major portion of the procedures is concerned.

5. A sample of crystalline antineuritic vitamin furnished by Dr. Jansen proved to be very effective (in conjunction with the unadsorbed factor) for the stimulation of the growth of certain yeasts. 1 part of the crystals in 100,000,000 parts of the culture medium can easily be detected. This crystalline substance has twice the potency of the most active preparation hitherto reported (Z_2).

6. However, one of the preparations made by Williams and Waterman from fractions discarded in the Jansen-Donath acetone-alcohol procedure has an activity for yeast at least twice as great as that of the crystalline vitamin. The antineuritic vitamin of Jansen and Donath thus appears to be *one* of the substances involved in yeast growth stimulation.

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THE BLOOD CHOLESTEROL FOLLOWING REPEATED ADMINISTRATIONS OF CHLOROFORM, PARALDEHYDE, AND URETHANE.

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WITH THE TECHNICAL ASSISTANCE OF C. BREWE.*

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(Received for publication, March 24, 1930.)

During the course of some experiments necessitating the repeated administration of chloroform to rabbits, an increase in fat in the Kupffer cells and capillaries of the liver was noted. Since usually the cholesterol increases are more or less parallel to the increase of fat and lecithin (1), a quantitative determination of the former substance was made. The amount of cholesterol in the blood was found to be far above normal.

With this finding in view, a series of experiments was instituted to determine the constancy and period of appearance of this hypercholesterolemia. Paraldehyde and urethane were administered both as a control to the chloroform and also as a parallel study in the possible general relationship between narcosis and hypercholesterolemia. The only report in the literature that had any bearing upon this work was that of Duccheschi (2) who administered chloroform by inhalation to dogs for from 1 to 1½ hours daily for several days. He found that the serum cholesterol increased in moderate degree during the first 2 to 3 days of narcosis and then diminished rapidly until it was reduced to unusually low values. His dogs lived only a few days. Our results do not agree with those of Duccheschi but the methods were somewhat different. Our period of experimentation was longer, and the animal we used, the rabbit, was a different one than the one he used, the dog.

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Methods.—The chloroform was administered to the rabbits in one of two ways, either by injection subcutaneously or by inhalation. By the former method, 0.5 cc. of chloroform was injected into the subcutaneous tissue of the groin and after a lapse of 1 week 0.25 to 0.5 cc. of chloroform was given in a similar way every other day. The great mortality of the animals receiving chloroform subcutaneously made it advisable to do most of the experiments by the inhalation method, although one rabbit did receive 34.2 cc. subcutaneously in a little over 9 weeks.

The animals which were given inhalations were anesthetized for ½ hour daily for a period of 2 weeks and then 1 hour daily for 2 more weeks. After a month they were placed, for 1 hour per day, in a large covered box which contained cotton saturated with chloroform. The period in the box was increased rapidly until they were kept there for 4 hours. By this means the rabbits were constantly in a chloroform atmosphere, and although they were only lightly anesthetized, still, an overdose of chloroform, to which rabbits are highly susceptible, was prevented. The box method has the important advantage of being time-saving.

Paraldehyde and urethane were given by stomach tube; 1 gm. per kilo being the dose used as advised by Sollmann (3).

The cholesterol was determined by the method described by Leiboff (4). Standards of different strengths were used and the unknown was read against the nearest standard. In every instance when a cholesterol determination was made upon the blood of an experimental animal, it was also made at the same time and under the same conditions upon the blood of one or more normal rabbits; thus the chemical method was constantly checked. The blood was usually withdrawn in the morning, before the next dose of chloroform was administered. The repeated withdrawal of blood did not affect the cholesterol. This was found experimentally. The quantity used was probably too small to cause any change.

Blood Cholesterol in Normal Rabbits.

The serum blood cholesterol in normal rabbits has been determined by Grigaut and coworkers (5) to average about 40 mg. per 100 cc. Weidman and Suderman (6) found it to vary between 64 and 80 mg. For whole blood Clarkson and Newburgh

(7) estimate 71.3 mg. per 100 cc. as the average, while Harnes (8) gives the range for his rabbits as from 51.1 to 73.3 mg. per cent. In our rabbits, both those used in these experiments and in many controls, the readings in blood serum cholesterol determinations upon 66 animals, ran between 19 and 100 mg. per 100 cc. with an average of 46.6 mg. per 100 cc.; most of the figures, however, were between 30 mg. and 80 mg. per 100 cc. Four results, namely 15, 19, 23, and 24 mg. per 100 cc. were below 30 mg. per 100 cc.; four—82, 83, 93, and 100 mg. were above 80 mg. per 100 cc. The wide variation, due to the yet imperfect methods available and to the greater difficulty that obtains in the rabbit whose blood cholesterol is low, was also experienced by Clarkson and Newburgh (7) and Harnes (8). In whole blood the former found the cholesterol of normal rabbits to vary between 35 and 125 mg. per cent, while the uncorrected figures of the latter run from 30 to 99.9 mg. per cent. It is evident then that only pronounced changes in the blood cholesterol can be considered of any interpretive value.

Blood Cholesterol in Chloroform Administration.

I. Subcutaneous Administrations.—Four animals were used in this series, three of whom had been given chloroform before blood cholesterol determinations were made. The fourth animal was studied rather carefully. See Table I.

The blood cholesterol values in the other rabbits were:

Rabbit 550; 240, 182, 232, and 193 mg.	Chloroform	administered	9 wks.
" 134; 137 mg.	"	"	19 days.
" 112; 119 "	"	"	1 mo.

II. Administration by Inhalation.—Eleven animals comprise this series. A typical protocol is given in Table II.

The blood serum determinations of the other rabbits gave the results shown in Table III.

Blood Cholesterol in Urethane Administration.[1]

Table IV indicates that urethane does not produce the rise in cholesterol that is found after repeated chloroform administration.

[1] Both urethane and paraldehyde were given every 5 to 7 days.

Except for one figure, 173 mg., the cholesterol readings are all practically within the range of the normal controls although it is

TABLE I.

*Cholesterol Determinations for Rabbit 593.**

Date.	Cholesterol.	Date.	Cholesterol.
	mg. per 100 cc.		*mg. per 100 cc.*
Apr. 21	74	May 23	220
May 8	73	" 26	175
" 15	110	June 3	140
" 19	150	July 7	156
" 21	190		

* On April 22, 0.5 cc. of chloroform was given subcutaneously, and commencing May 8, 0.5 cc. of chloroform was given every 2 days.

TABLE II.

Rabbit 473.

Date.	Length of exposure to chloroform.	Cholesterol determination.	Date.	Length of exposure to chloroform.	Cholesterol determination.
	hrs.	*mg. per 100 cc.*		*hrs.*	*mg. per 100 cc.*
Mar. 18	½	40	Apr. 28	2	100
" 19	½	11	May 5	4	230
" 25	½	68	" 13	4	100
" 31	½	42	" 24	4	170
Apr. 11	1	73	" 26	4	145

TABLE III.

Blood Serum Determinations on Rabbits.

Rabbit No.	Normal cholesterol.	Cholesterol determination.	Rabbit No.	Normal cholesterol.	Cholesterol determination.
	mg. per 100 cc.	*mg. per 100 cc.*		*mg. per 100 cc.*	*mg. per 100 cc.*
79	33	147 after 5 mos.	713	34	114 after 1 mo.
475	43	180 " 10 wks.	187	81	163 " 2 mos.
966	74	120 " 4 mos.	189		121 " 2 "
172	46	133 " 2 "	51		166 " 10 wks.
188	78	163 " 1 mo.	53		179 " 11 "

interesting to note that in this series the average of the normal readings gives 48.4 mg. whereas the average of the readings in the

animals who had received more than two doses of urethane is 85 mg. per 100 cc. This might possibly be interpreted as a slight rise.

Blood Cholesterol in Paraldehyde Administration.

In these experiments the number of animals are fewer and the results are less definite. (See Table V.)

TABLE IV.

Blood Cholesterol after Urethane Administration.

Rabbit No.	Normal cholesterol.	Cholesterol determination.	
	mg. per 100 cc.	*mg. per 100 cc.*	
397	Too low.	94 after 7 doses,	56 after 16 doses.
603	30.3	85 " 6 "	
601	36.3	98 " 6 "	173 after 14 doses.
602	69.4	96 " 7 "	
395	68.9	59 " 7 "	
934		80 " 2 "	
645	37	73 " 6 "	54 after 14 doses.
936		54 " 2 "	
710		71 " 5 "	62 after 13 doses.
711		102 " 5 "	

TABLE V.

Blood Cholesterol after Paraldehyde Administration.

Rabbit No.	Normal cholesterol.	Cholesterol determination.	
	mg. per 100 cc.	*mg. per 100 cc.*	
735	24	79 after 6 doses.	
734	83	56 " 4 "	
103	82	68 " 4 "	
938		74 " 3 "	
742		40 " 2 "	105 after 8 doses.
743		40 " 2 "	40 " 8 "
939		56 " 2 "	
937		62 " 2 "	

On the whole the indications are that repeated administration of paraldehyde does not produce an increase in blood cholesterol.

SUMMARY AND CONCLUSIONS.

There appears to be a definite rise in blood cholesterol beginning about 3 weeks after repeated administration of chloroform. If

the chloroform administration is continued for a longer period, the cholesterol level remains high, but does not show a progressive rise. No very marked change in the blood cholesterol occurs after repeated administration of urethane or paraldehyde, although we cannot, at the present time, rule out the possibility that a very slight increase in cholesterol takes place after repeated urethane administrations.

As to the significance of the increase in cholesterol in the blood under those conditions no definite statement can be made. Further investigations are being carried out to determine this.

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THE APPARENT DISSOCIATION CONSTANT OF GLYCINE ETHYL ESTER.*

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(Received for publication, April 19, 1930.)

According to the classical theory of the dissociation of weak electrolytes, the amino acids show much lower basic and acidic dissociation constants than would be expected from a comparison with other weak acids and bases. In order to explain this anomaly, Adams (1) originally suggested that an ampholyte must necessarily exist almost entirely as an "inner salt." Bjerrum (2) extended this idea with additional evidence, and adopted the term "Zwitterion" in place of the "inner salt" of Adams. The evidence in favor of this hypothesis has been extended by others, notably Harris (3) and Borsook.[1]

The attempts to prove the Adams and Bjerrum hypothesis have largely centered on formol titration methods in which the basic properties of the amino group are reduced. Equally valid as evidence would be a corresponding titration of the amino group after destruction of the acidic properties of the carboxyl groups. This can be accomplished by formation of the ester of the amino acid. Since accurate data on the basic dissociation constants of esters of the amino acids seem to be lacking, this investigation was undertaken to partially remedy this deficiency. Veley (4), using a colorimetric method, reports a value of K_b' for glycine ethyl ester of 9.7×10^{-8}. Johnston (5), using a catalytic method, states that this value is higher than 2.2×10^{-8}.

* Aided in part by a grant from the Chemical Foundation, Incorporated, to Professor Carl L. A. Schmidt.

[1] Private communication.

EXPERIMENTAL.

Glycine ethyl ester hydrochloride was prepared by passing HCl gas through a mixture of absolute alcohol and glycine. The ester

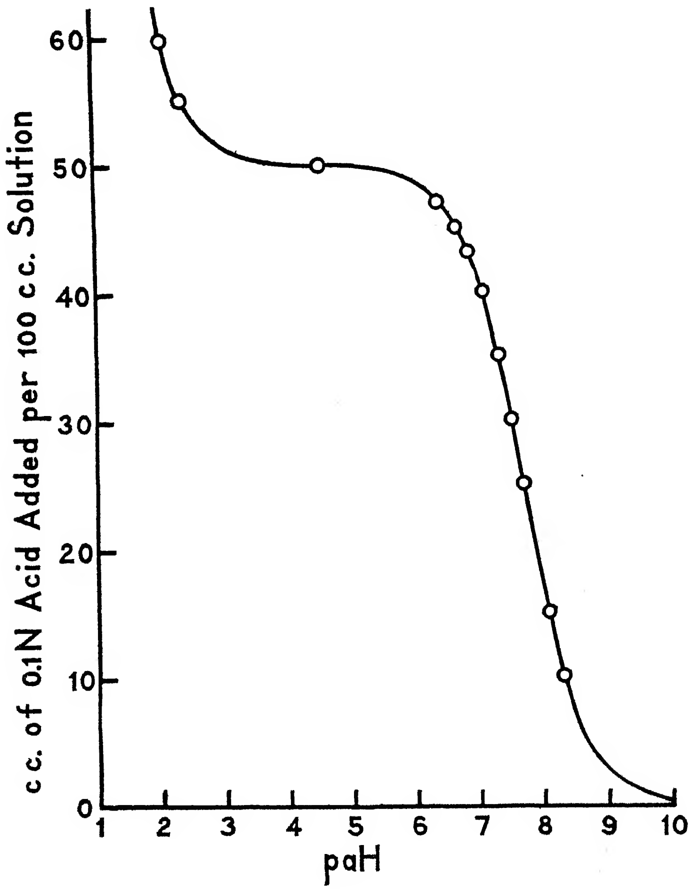

FIG. 1. Titration curve of 0.05 M glycine ethyl ester.

hydrochloride so prepared was recrystallized three times from absolute alcohol, dried, and analyzed for nitrogen by the Kjeldahl method. The per cent of nitrogen calculated is 10.04 per cent; found, 10.02, 10.04 per cent.

The titration curve was obtained by use of the hydrogen and quinhydrone electrodes at 25° in the same manner as previous titration curves determined in this laboratory (6). The hydrochloride was titrated with standard base and acid from its original paH, rather than the more usual procedure of using the free base as starting material. Since the ester is subject to hydrolysis, particularly in alkaline solution, it was desirable to measure the paH as rapidly as possible. This difficulty was less important than was anticipated since the hydrolysis in the solutions measured did not produce a measurable change in paH within an hour. The data are shown in Fig. 1. The points represent experimental data. The curve, on the other hand, is a theoretical one, constructed by plotting points calculated from the Henderson-Hasselbalch equation.

$$\text{paH} = \text{pK}_w - \text{pK}_b' + \log \frac{(\text{BOH})}{(\text{B}^+)}$$

The agreement is entirely satisfactory and shows that hydrolysis of the ester is a factor of no importance. Measurements were not made in solutions having a higher paH than 8.12, in order to avoid the possibility of complication by increased hydrolysis in the more alkaline solutions.

The apparent dissociation constant, K_b' was found to be 5.37 $\times 10^{-7}$; p$K_b' = 6.27$.

DISCUSSION.

The classical apparent dissociation constant K_b' of glycine is 2.6×10^{-12}. The "Zwitterion" constant defined as

$$K_B' = \frac{K_w}{K_a'}$$

is 5.55×10^{-5}. The constant obtained for glycine ester is obviously in better agreement with the "Zwitterion" constant than with the classical constant. A considerable deviation from the "Zwitterion" constant is to be expected, since the effect of —COOR on the dissociation of $—NH_3^+$ is very different from the effect of the negatively charged group $—COO^-$. If, on the other hand, we consider the glycine molecule to be undissociated according to the classical theory, rather than as a "Zwitterion," the effect of

—$COOC_2H_5$ on the dissociation of —NH_3^+ would approximate the effect of —COOH. Thus the deviation of the ester K_b' from that of glycine would be slight, on the basis of classical theory. Since this deviation from the classical K_b' is very large, but the deviation from the "Zwitterion" constant no greater than would be expected, these data may be considered as evidence supporting the "Zwitterion" hypothesis of Adams and Bjerrum.

CONCLUSION.

The apparent dissociation constant, K_b', of glycine ethyl ester has been found to be 5.37×10^{-7}.

The significance of this value is better interpreted in terms of the "Zwitterion" hypothesis of Adams and Bjerrum than in terms of the classical theory of dissociation.

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THE HYDROGENATION OF UNSATURATED LACTONES TO DESOXY ACIDS.

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(Received for publication, April 26, 1930.)

During the past year, by a rather curious coincidence, reports of similar experiences have appeared from four different laboratories where work was carried out in distinct fields. These have had to do with the cleavage of certain lactones to desoxy acids on catalytic hydrogenation. The first of these was the report of Borsche and Peitzsch[1] on the catalytic reduction of methysticin to tetrahydromethysticinic acid through the intermediate lactone dihydromethysticin. Simultaneously there appeared a report of Mannich and Butz[2] on the hydrogenation of several unsaturated δ-lactones, which led through the intermediate saturated lactone to the desoxy acid. Somewhat later La Forge and Smith[3] described their observations on the hydrogenation products of rotenone. The latter was found to yield on addition of 2 atoms of hydrogen principally two isomeric substances, a neutral dihydrorotenone and an acid, rotenonic acid. In the latter case the acid was produced by cleavage of the lactone group to the desoxy acid and appeared to be independent of the hydrogenation of the double bond.[4]

Before the appearance of the above reports Jacobs and Gustus had already met with and later published[5] a similar experience in the

[1] Borsche, W., and Peitzsch, W., *Ber. chem. Ges.*, **62**, 360 (1929).

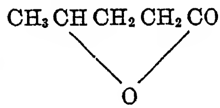
[2] Mannich, C., and Butz, A., *Ber. chem. Ges.*, **62**, 461 (1929).

[3] La Forge, F. B., and Smith, L. E., *J. Am. Chem. Soc.*, **51**, 2574 (1929); **52**, 1088 (1930).

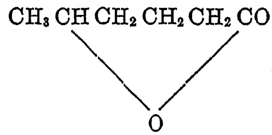
[4] The cleavage of the furane ring in another type of substance on catalytic hydrogenation has been presented by Kaufmann, W. E., and Adams, R., *J. Am. Chem. Soc.*, **45**, 3029 (1923).

[5] Jacobs, W. A., and Gustus, E. L., *J. Biol. Chem.*, **86**, 203 (1930).

case of the hydrogenation with various catalysts of the unsaturated lactone anhydro-γ-isogitoxigenic methyl ester to the saturated desoxy acid, γ-digitoxanoldiacid monomethyl ester following the absorption of 2 mols of hydrogen. In this case all efforts to intercept the reaction at the 1 mol stage in order to obtain the saturated lactone by hydrogenation of the double bond alone failed. The only product of the reaction observed was the saturated desoxy acid and unchanged starting material. Since in the case of a somewhat analogous saturated lactone, γ-isodigitoxigenic acid (methyl ester), all attempts to accomplish a similar lactone cleavage had failed, the unique behavior of the unsaturated lactone mentioned above suggested along with other previously discussed observations that the double bond in this substance is associated with the lactone ring. In view of the importance of this conclusion for the determination of certain points in the structure of gitoxigenin, it was decided to investigate the behavior of simpler unsaturated lactones on catalytic hydrogenation. This study has yielded rather interesting results.



I.



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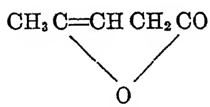
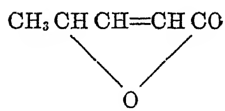
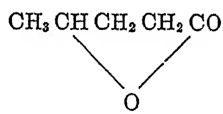
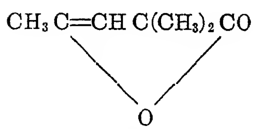
 ⟶ $CH_3CH_2CH_2CH_2COOH$

III.

IV.

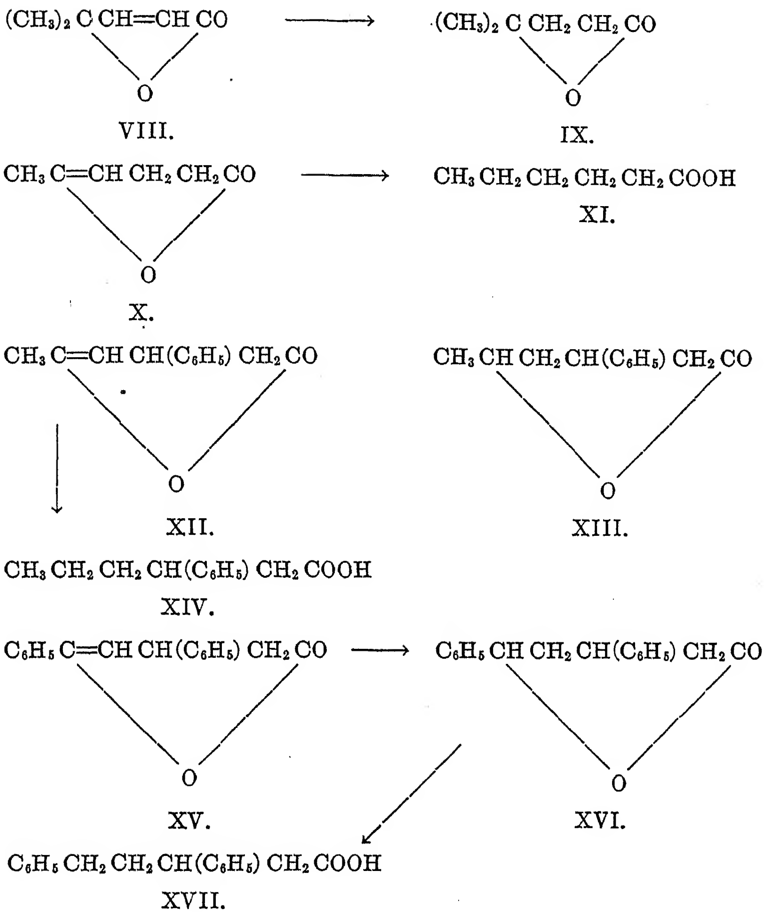
 ⟶ 

V.

 ⟶ $CH_3CH_2CH_2C(CH_3)_2COOH$

VI.

VII.



A comparison of the behaviors of $\Delta^{\beta,\gamma}$- and $\Delta^{\alpha,\beta}$-angelicalactone has shown a striking contrast. In repeated experiments $\Delta^{\beta,\gamma}$-angelicalactone (Formula III) was found to absorb 2 mols of catalytically activated hydrogen very readily with the formation of valeric acid (Formula IV). On the other hand, the $\Delta^{\alpha,\beta}$-isomer (Formula V) was found to take principally a different course and stopped at approximately the 1 mol stage with the production of γ-valerolactone (Formula I). In this case only approximate figures for 1 mol were obtained and in different experiments a varying amount of excessive absorption was noted with a corre-

sponding formation of valeric acid. But this excessive absorption occurred during the main reaction and once prompt absorption had stopped, no further appreciable consumption of hydrogen could be detected. The relatively small formation of valeric acid, we believe, is attributable to the presence of the $\Delta^{\beta,\gamma}$- isomer. It is well known that the two isomers readily undergo interconversion during certain reactions. During the hydrogenation a portion of the $\Delta^{\alpha,\beta}$-angelicalactone must rearrange to the $\Delta^{\beta,\gamma}$ form and in an amount which may vary with temperature conditions and the rapidity with which hydrogenation proceeds. Repeated attempts to cause such a cleavage of γ-valerolactone itself to valeric acid were unsuccessful. In such experiments subsequent addition of $\Delta^{\beta,\gamma}$-angelicalactone to the mixture resulted in prompt quantitative hydrogenation of the latter to valeric acid and the unattacked valerolactone was readily recovered as such. In view of the comparative instability of $\Delta^{\beta,\gamma}$-angelicalactone and its ready saponification to levulinic acid, it appeared possible that its peculiar behavior might be due to the intermediate formation of the keto acid which might be reduced to valeric acid. This seemed excluded, however, because in a direct study of the behaviour of levulinic acid itself under the conditions of hydrogenation, it was found to absorb hydrogen very slowly and in a manner not comparable with $\Delta^{\beta,\gamma}$-angelicalactone.

In order to make more clear cut the difference in behavior of the two types of unsaturated γ-lactones we have selected two substances in which the double bond is fixed without the possibility of a shift within the lactone ring, namely α,α-dimethyl-$\Delta^{\beta,\gamma}$-angelicalactone (Formula VI) and γ-methyl-$\Delta^{\alpha,\beta}$-angelicalactone (Formula VIII). In the former case a prompt hydrogenation to the desoxy acid, α, α-dimethyl-n-valeric acid occurred while in the case of the $\Delta^{\alpha,\beta}$-lactone only 1 mol of hydrogen was absorbed with the formation of γ-methyl-γ-valerolactone. We have then turned to the δ-lactones with similar results. As in the case of γ-valerolactone, all attempts to cleave δ-n-caprolactone were unsuccessful. On the other hand, the lactone of δ-hydroxy-$\Delta^{\gamma,\delta}$-hexenoic acid (Formula X) promptly absorbed 2 mols of hydrogen with the formation of n-caproic acid.

From these results it appears that unsaturated lactones in which the double bond is attached to the point of lactonization, as in the

lactones of enolized keto acids, are reduced to the desoxy acid on catalytic hydrogenation. The results of Mannich and Butz with the lactone of δ-hydroxy-β, δ-diphenyl-$\Delta^{\gamma,\delta}$-pentenoic acid (Formula XV) and an analogous β-(methylenedioxyphenyl) derivative are in conformity with these results. However, their experience with the lactone of δ-hydroxy-β-phenyl-$\Delta^{\gamma,\delta}$-hexenoic acid (Formula XII) appeared to present an exception. Since they have reported that their substance failed to absorb hydrogen at all, it appeared to us that the failure might be attributable to manipulative complications rather than to the structure of the lactone. We have therefore reexamined the behavior of this substance using a different solvent and catalyst and found, contrary to Mannich and Butz, that it readily absorbs 2 mols of hydrogen with the formation of a desoxy acid and therefore presents a normal behavior. Their suggestion is, therefore, superfluous, that in the case of the phenyl-substituted unsaturated lactones which may be regarded as derivatives of substituted benzyl alcohols their hydrogenation to the desoxy acids is analogous to the reduction of benzyl alcohol to toluene. In view of the observation of these workers, however, that the hydrogenation of the diphenyl lactone (Formula XV) when interrupted at the 1 mol stage gives a good proportion of the saturated lactone (Formula XVI), which was therefore unquestionably the intermediate stage in the formation of the desoxy acid, it was of interest to determine whether the same situation would be met in the case of the hexenoic lactone (Formula XII). This was found, however, not to be the case at least by our procedure, since on interruption of the hydrogenation at the 1 mol stage about half of the reaction product proved to be the desoxy acid, and an approximately corresponding amount of starting material was found to be unattacked. If the saturated lactone was formed in this case as the intermediate stage, the amount in which it persisted was so small as to make its certain identification difficult. In the present studies we have consistently employed the same procedure throughout, namely alcohol as solvent and the platinum oxide of Adams and Shriner as catalyst. This procedure differed from that used by Mannich and Butz and the question is left open for the present whether the difference in behaviors of the diphenyl lactone (XV), as reported by them, and of the hexenoic lactone (XII) in our hands is attributable to this difference in procedure or to the substances themselves.

In the case of methysticin, according to Borsche and Peitzsch, the principal reaction is a prompt hydrogenation to dihydromethysticin, but accompanying this there is a simultaneous formation of about 10 per cent of the desoxy acid. They have apparently shown that the latter proceeds through the dihydro stage by the fact that dihydromethysticin may be transformed also, although far more slowly, into the desoxy acid. They have suggested as a possible explanation of the greater speeds of acid formation in the former case that the free energy developed during hydrogenation of the double bond of methysticin may accelerate the otherwise slow tendency to further reductive cleavage of the lactone ring. It is possible that this principle may more strongly govern in the case of lactones of enolized keto acids. Here the double bond is attached to the same carbon atom upon which lactonization has occurred so that the second reaction, *i.e.* cleavage to the desoxy acid, may be so accelerated as to keep pace with hydrogenation of the double bond. This is so marked that the saturated lactone which might be formed could not be readily isolated as such. This was substantiated by an experiment in which on hydrogenation of a mixture of $\Delta^{\beta,\gamma}$-angelicalactone and γ-valerolactone the hydrogen absorbed and the acidity developed corresponded quantitatively with the amount of the former, and valerolactone was recovered unchanged. It is apparent that in certain cases, such as the lactones of Mannich and Butz, the factors involved are not so simple. Here the intermediate saturated lactones in appreciable amounts may be isolated as such, and may be in turn easily cleaved to the desoxy acids.

In summing up our experience, it appears that wherever a substance containing a lactone group and a double bond is promptly hydrogenated to the saturated desoxy acid without a definite break at the 1 mol stage with formation of the saturated lactone it may be taken as strongly presumptive evidence that one is dealing with the lactone of an enolized oxo-acid. These observations support the interpretation given in our former work to the behavior on hydrogenation of anhydro-γ-isogitoxigenic methyl ester where the saturated lactone γ-isogitoxigenic methyl ester was unattacked by catalytically activated hydrogen. The former is possibly a $\Delta^{\gamma,\delta}$ unsaturated δ-lactone.

There is, however, a point which requires further investigation,

namely the behavior of the cardiac aglucones themselves on hydrogenation. In previous reports from this laboratory and from those of Windaus and Cloetta of experiments on hydrogenation, the formation only of the neutral saturated lactones has been noted. The possible production of any acid products has not been suggested. Since we have found that these substances are $\Delta^{\beta,\gamma}$ unsaturatd γ-lactones, it is strange that their hydrogenation has not continued to the desoxy acids. This problem is being studied further in order to determine, if possible, the reason for such an apparently anomalous behavior.

EXPERIMENTAL.

In the following experiments a constant procedure was used in which the substances were hydrogenated in ethyl alcoholic solution with platinum oxide catalyst prepared according to Adams and Shriner.

Hydrogenation of $\Delta^{\beta,\gamma}$-Angelicalactone.—This lactone was prepared by the method of Thiele, Tischbein, and Lossow[6] and a middle fraction which gave a b.p. of 55–56° at 12 mm. pressure was employed. A solution of 0.71 gm. of this fraction in ethyl alcohol was treated with 0.05 gm. of platinum oxide catalyst and shaken with hydrogen. The absorption of hydrogen proceeded rapidly and came to a stop after 15 minutes. However, observation was continued for several hours but no appreciable additional absorption could be detected. After correcting for the reduction of the catalyst the observed hydrogen absorption was found to be 325 cc. (760 mm., 0°).[7] Calculated for 2 mols of H_2, 325 cc. The reaction mixture smelled strongly of valeric acid and was acid in reaction. In this experiment the substance was not isolated.

In a second experiment under identical conditions, 1.05 gm. of $\Delta^{\beta,\gamma}$-angelicalactone with 0.05 gm. of catalyst were found to absorb 487 cc. of hydrogen within 30 minutes. Calculated for $2H_2$, 480 cc.

The free acid present in the alcoholic reaction mixture after filtering from catalyst was determined by direct titration against phenolphthalein with 0.1 N sodium hydroxide solution. Calculated for 100 per cent conversion to valeric acid, 107 cc. Found, 97 cc.

[6] Thiele, J., Tischbein, R., and Lossow, E., *Ann. Chem.*, **319**, 184 (1901).

[7] All gas volumes are given for the dry gas at 0° and 760 mm.

The presence of valeric acid was confirmed by the preparation of the silver salt. For this purpose the alkaline mixture was concentrated under diminished pressure to remove the alcohol. The solution of the residue in a few cc. of water was carefully neutralized with dilute sulfuric acid and was then treated with 10 per cent silver nitrate solution. Recrystallization of the salt from water gave white needles which were dried for analysis at 60° and 15 mm. over $CaCl_2$.

5.776 mg. substance: 2.270 mg. H_2O, 6.124 mg. CO_2, 2.966 mg. Ag.
$C_5H_9O_2Ag$. Calculated. C 28.71, H 4.35, Ag. 51.63.
Found. " 28.91, " 4.39, " 51.35.

Hydrogenation of $\Delta^{\alpha,\beta}$-Angelicalactone.—The $\Delta^{\alpha,\beta}$-angelicalactone (b.p. 84.5°, 12 mm.) in 0.2 per cent solution did not reduce ammoniacal silver solution. 3.21 gm. were hydrogenated with 0.06 gm. of platinum oxide. The reaction definitely stopped after 74 minutes. The calculated absorption for 1 mol of H_2 is 733 cc. Found, 795 cc.

That this excessive absorption over the 1 mol requirement indicated the formation of a small amount of valeric acid was confirmed by the titration experiments. Direct titration against phenolphthalein showed the presence of free acid corresponding to 5.07 cc. of N sodium hydroxide solution. 50 cc. of N alkali were then added and after refluxing for 1 hour the mixture was titrated back. The additional alkali consumed by saponification of valerolactone was 27.5 cc. of N sodium hydroxide. The total calculated alkali requirement is 32.8 cc. Found, 32.6 cc.

The formation of γ-valerolactone was confirmed by its isolation as follows. The above alkaline titration mixture was concentrated to dryness. A concentrated solution of the residue was strongly acidified to Congo red with dilute sulfuric acid, and the solution was refluxed for 30 minutes to insure lactonization. After extraction with ether and washing the extract with 50 per cent potassium carbonate solution the extract was dried with ignited potassium carbonate. Fractionation gave γ-valerolactone which boiled at 16 mm. at 87–90°.

4.093 mg. substance: 2.940 mg. H_2O, 8.986 mg. CO_2.
$C_5H_8O_2$. Calculated. C 59.96, H 8.06.
Found. " 59.88, " 8.04.

The course of the hydrogenation was verified by a second experiment conducted in a similar manner. The fact that the lactone had been permitted to stand for 8 days before use with the possibility of appreciable isomerization probably accounts for the somewhat larger relative absorption of hydrogen beyond the 1 mol stage with a corresponding formation of a greater amount of valeric acid.

2.99 gm. of lactone and 0.05 gm. of catalyst were used. Calculated absorption for 1 mol of H_2, 683 cc. Found, 817 cc.

5.3 cc. of N sodium hydroxide solution were required to neutralize the free acidity and an additional 24.7 cc. for saponification or a total of 30 cc. Calculated, 30.5 cc.

The γ-valerolactone recovered boiled at 90–91° at 16 mm.

5.947 mg. substance: 4.315 mg. H_2O, 13.050 mg. CO_2. Found. C 59.85, H 8.11.

Attempted Hydrogenation of γ-Valerolactone.—1.28 gm. of γ-valerolactone, b.p. 96–100° at 29 mm., in ethyl alcoholic solution were shaken with hydrogen and 0.05 gm. of catalyst. After the prompt reduction of the latter, no further absorption of hydrogen was noted even after 20 hours, although an additional portion of catalyst had been added after the 1st hour. Subsequent addition of 0.59 gm. of $\Delta^{\beta,\gamma}$-angelicalactone to the mixture resulted in a prompt addition within 3 hours of 2 mols of H_2 based on the angelicalactone used. Found, 264 cc. Calculated, 270 cc.

In a second experiment a mixture of the two lactones was used at the start. 2.16 gm. of γ-valerolactone and 0.71 gm. of $\Delta^{\beta,\gamma}$-angelicalactone were treated in the usual manner with 0.05 gm. of catalyst. Hydrogenation ceased abruptly at the end of 30 minutes. The observed hydrogen absorption was 315 cc. Calculated for the $\Delta^{\beta,\gamma}$-angelicalactone present, 324 cc.

Titration of the free acid in the reaction mixture confirmed the conversion of the unsaturated lactone to valeric acid, while the saturated lactone remained unchanged. Direct titration required 6.6 cc. of N sodium hydroxide solution. Calculated for valeric acid formed from 0.71 gm. of angelicalactone, 7.2 cc. The titration mixture was then boiled with an excess of N alkali and again titrated back. Calculated for the valerolactone originally used, 21.6 cc. Found, 20.5 cc.

The titration mixture after concentration was relactonized as previously given and γ-valerolactone was recovered. It boiled at 17 mm. at 86–87°.

5.910 mg. substance: 4.380 mg. H_2O, 12.920 mg. CO_2. Found. C 59.62, H 8.29.

Hydrogenation of α,α-Dimethyl-$\Delta^{\beta,\gamma}$-Angelicalactone.—This lactone, which was prepared according to Pinner,[8] boiled at 18 mm. at 59° and formed long, transparent prisms when cooled in ice.

0.330 gm. of the lactone with 0.015 gm. of catalyst absorbed within 22 minutes 118 cc. of hydrogen. Calculated for $2H_2$, 117 cc.

In another experiment, 1.69 gm. of lactone and 0.05 gm. of catalyst were employed. For some undetermined reason (possibly due to poisoning of the catalyst), the absorption of hydrogen was unusually slow. This came to a stop after 18 hours. The observed absorption was 591 cc. Calculated for $2H_2$, 601 cc.

The filtered reaction mixture on titration required for neutralization 11.5 cc. of N sodium hydroxide solution. The calculated requirement was 13.4 cc. From the concentrated solution the silver salt of α,α-dimethylvaleric acid was prepared which formed needles after recrystallization from water.

6.430 mg. substance: 3.235 mg. H_2O, 8.367 mg. CO_2, 2.923 mg. Ag.

$C_7H_{13}O_2Ag$.	Calculated.	C 35.45,	H 5.54,	Ag 45.53.
	Found.	" 35.50,	" 5.63,	" 45.46.

Hydrogenation of γ-Methyl-$\Delta^{\alpha,\beta}$-Angelicalactone.—Terelactone was prepared from the dibromide of pyroterebic acid by boiling with aqueous sodium carbonate solution. The pyroterebic acid necessary as an intermediate was readily obtained from its isomer by the method of Goldberg and Linstead[9] in which $\Delta^{\alpha,\beta}$-isohexenoic acid is prepared by the Doebner reaction and converted to the isomer, pyroterebic acid, by heating with alkali. The terelactone was a colorless, neutral liquid which boiled at 72 mm. at 127°.

0.59 gm. of terelactone and 0.05 gm. of catalyst showed a complete absorption of 1 mol of hydrogen within 7 minutes. On continued shaking for 20 hours more, no appreciable additional

[8] Pinner, A., *Ber. chem. Ges.*, **15**, 579 (1882).
[9] Goldberg, A. A., and Linstead, R. P., *J. Chem. Soc.*, 2354 (1928).

absorption was noted. The observed absorption was 107 cc. Calculated for 1 mol of H_2, 118 cc.

The free acid in the mixture was not appreciable and only 0.19 cc. of N alkali was required for neutralization. On saponification with an excess of alkali an additional 4.9 cc. of N alkali were consumed, or a total of 5.1 cc. Calculated, 5.3 cc. The initial slight acidity was possibly due to slight, spontaneous opening of the lactone to the hydroxy acid. From the above saponification mixture after concentration the silver salt of γ-hydroxyisocaproic acid was prepared in the usual manner and formed lustrous needles from water.

5.640 mg. substance: 2.380 mg. H_2O, 6.223 mg. CO_2, 2.530 mg. Ag.

$C_6H_{11}O_3Ag$.	Calculated.	C 30.13, H 4.64, Ag 45.15.
	Found.	" 30.09, " 4.72, " 44.86.

Hydrogenation of the Lactone of δ-Hydroxy-$\Delta^{\gamma,\delta}$-Hexenoic Acid.—This lactone was prepared by the the method of Vorländer and Knötzsch[10] and showed a b.p. of 112–115° at 69 mm.

0.625 gm. of the lactone and 0.05 gm. of catalyst gave a completed reaction corresponding to 2 mols of H_2 in 30 minutes. The absorption was 242 cc. Calculated, 250 cc.

On direct titration of the free acidity 5.2 cc. of N alkali were required. Calculated, 5.6 cc. The silver salt of caproic acid was prepared in the usual manner.

6.190 mg. substance: 2.800 mg. H_2O, 7.370 mg. CO_2, 2.990 mg. Ag.

$C_6H_{11}O_2Ag$.	Calculated.	C 32.39, H 4.98, Ag 48.39.
	Found.	" 32.47, " 5.06, " 48.30.

Hydrogenation of the Lactone of δ-Hydroxy-β-Phenyl-$\Delta^{\gamma,\delta}$-Hexenoic Acid.—This lactone was prepared by the method of Vorländer and Knötzsch[10] and showed a b.p. of 167–170° at 12 mm. 0.865 gm. was hydrogenated with 0.1 gm. of catalyst. After 20 minutes the reaction showed a decided change in the rate of absorption and then proceeded slowly. After an additional 20 minutes the reaction was interrupted since the continued slow absorption was probably due to reduction of the benzene nucleus. The observed absorption was 269 cc. Calculated for 2 mols of

[10] Vorländer, D., and Knötzsch, A., *Ann. Chem.*, **294**, 317 (1897).

H_2, 206 cc. To neutralize the free acid developed, 37 cc. of N alkali were required. On subsequent saponification with excess alkali an additional 8 cc. were consumed, or a total of 45 cc. possibly due to some ester formation during the hydrogenation. The calculated alkali equivalent for the original lactone is 47 cc.

The silver salt of the resulting β-phenyl-*n*-caproic acid was obtained as usual for its identification.

4.925 mg. substance: 2.390 mg. H_2O, 8.720 mg. CO_2, 1.780 mg. Ag.

$C_{12}H_{15}O_2$. Ag.	Calculated.	C 48.16, H 5.05, Ag 36.09.
	Found.	" 48.29, " 5.43, " 36.15.

In another experiment the hydrogenation was interrupted after an alcoholic solution of 8.5 gm. of lactone with 0.05 gm. of catalyst had absorbed 1013 cc. or 1 mol of H_2. The alcoholic solution after filtration from catalyst was concentrated, the residue was taken up in ether, and the solution was extracted with aqueous potassium carbonate. Ether extraction of the acidified carbonate solution gave on concentration 4.5 gm. of crude acid as an oil. On fractionation the major portion distilled at 179–181° at 20 mm. and was found by analysis to be β-phenyl-caproic acid.

3.080 mg. substance: 2.305 mg. H_2O, 8.468 mg. CO_2.

$C_{12}H_{16}O_2$.	Calculated.	C 74.96, H 8.38.
	Found.	" 74.98, " 8.37.

The above ether solution containing the neutral fraction was concentrated and the residue, which consisted mostly of unchanged, unsaturated lactone, was converted into the acid by saponification with an excess of aqueous N sodium hydroxide solution. After reacidifying strongly with hydrochloric acid and refluxing for 30 minutes to relactonize any β-phenyl-δ-hydroxy-caproic acid which might have been present, the mixture was again extracted with ether. The β-phenyl-δ-keto-caproic acid which could not relactonize under these conditions was recovered by extraction of the ether solution with carbonate. Acidification of the carbonate solution gave 3.5 gm. of the keto acid in characteristic needles which melted at 83°.

The neutral material which remained after the above carbonate extraction of the keto acid and concentration of the ether solution weighed approximately 0.2 gm. This was an amount too small for

direct purification for analysis. An attempt to prepare a silver salt after saponification was unsuccessful. The possibility remains, however, that this small amount of material may have been saturated β-phenyl-δ-caprolactone which may represent the intermediate stage in the formation of the desoxy acid or may have been formed from a small amount of an isomer of the original unsaturated lactone in which the double bond has shifted from the position γ, δ.

THE NATURE OF VITAMIN C.*

A STUDY OF ITS ELECTRICAL TRANSFERENCE.

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INTRODUCTION.

The object of this investigation has been to study the acid-basic function of vitamin C by means of electrical transference methods.

The instability of vitamin C (1) results in loss of the antiscorbutic value of foods and makes the study of the vitamin highly desirable, but the same property makes experimental work very difficult. Destruction is due chiefly to oxidation and is favored by heat, catalysts, and alkalinity (2).

If in an acid solution, an electric current transfers the vitamin toward the cathode, the vitamin is either a true base or an ampholyte, but if transferal takes place toward the anode it can only be a true acid. If the solution is basic and migration is toward the cathode then the vitamin is a true base, but if toward the anode it can either be a true acid or an ampholyte. During a simple electrolysis, the conditions at either electrode would be ideal for inactivation, because of alkali being liberated at the cathode, oxygen and chlorine at the anode, and heat being developed throughout the solution. It is thought that the apparatus devised, and the features involved may be of service in other problems.

EXPERIMENTAL.

Fig. 1 shows the cell, composed of three main parts, an anode compartment, a cathode compartment, and a middle section. The

* Contribution No. 184 from the Department of Chemistry, University of Pittsburgh.

electrode compartments are porous alundum cups. The electrodes are identical, each being a platinum wire protected by an alundum tube, which is kept under a slight suction and through which a

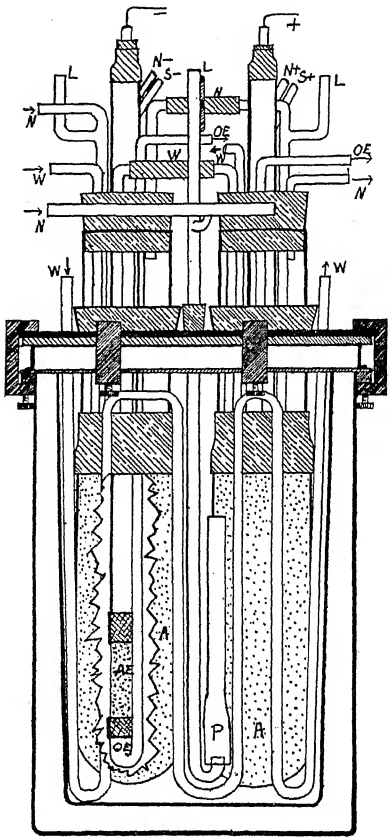

Fig. 1. The cell. A represents the porous alundum cup; AE, alundum tube covering electrode; L, tubes for adding solution to be electrolyzed; N, nitrogen to cell; N^-, N^+, nitrogen, through electrodes; OE, outlet of electrodes for sodium chloride solution, nitrogen, etc.; P, circulator run by nitrogen; S^-, S^+, sodium chloride solution, to electrodes; W, water.

stream of sodium chloride solution and nitrogen gas circulates. The suction prevents diffusion of harmful materials out of the tube and the salt solution maintains conduction, and with the nitrogen

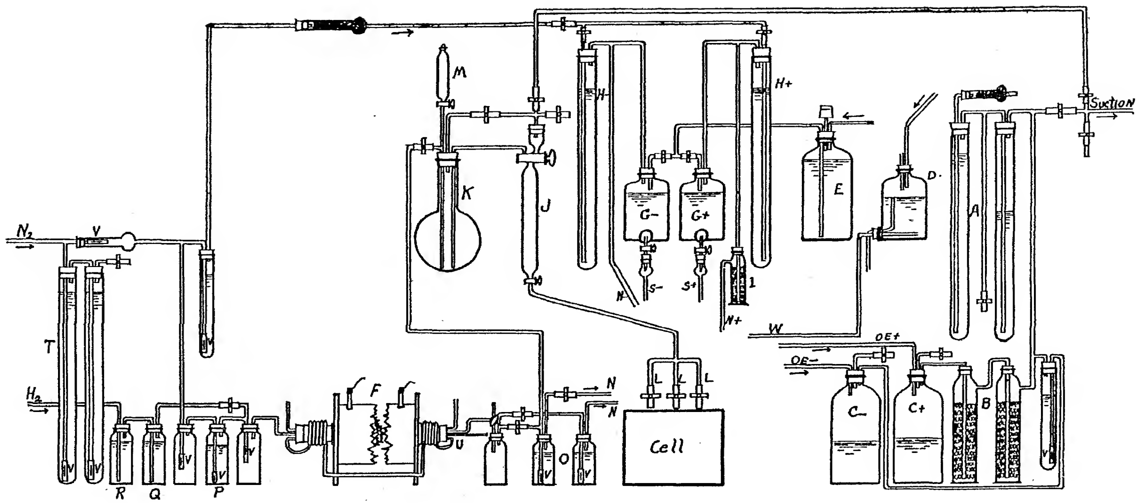

Fig. 2. Apparatus related to cell. A represents suction regulators; B, soda-lime towers on suction line to absorb chlorine; C^-, C^+, receivers for solution from electrode; D, water pressure regulator; E, reserve reservoir for sodium chloride solution; F, special furnace to remove oxygen from nitrogen; G^-, G^+, sodium chloride solution containers; H^-, H^+, regulators to control the flow of nitrogen and sodium chloride solution through electrodes and also to regulate suction at the electrode; I, soda-lime tower to keep chlorine out of the sodium chloride solution and the regulator; J, measuring tube for lemon juice solution; K, flask for mixing solution in anaerobic condition; L, lemon juice solution to cell; M, funnel for adding ammonia; N, nitrogen to cell; N^-, N^+, nitrogen through electrodes; O, bubble counters and washers for nitrogen; OE^-, OE^+, sodium chloride solution, etc. from electrodes; P, alkaline pyrogallol; Q, alkali; R, alkaline potassium permanganate; S^-, S^+, sodium chloride solution to electrodes; T, pressure regulator on nitrogen supply line; U, cooling coils and thermometer on furnace; V, one-way valves; W, water inlet to cell.

rapidly carries away the liberated alkali, oxygen or chlorine, in addition to much of the heat. Cooling coils are placed in all three sections. The cell is air-tight and to insure this, a stream of pure nitrogen is circulated under slight pressure through all parts. The oxygen is removed from ordinary tank nitrogen by first passing through alkaline pyrogallol and then through a special furnace containing a core of freshly reduced copper oxide, 32 inches long and 2 inches in diameter. Fig. 2 shows the arrangement of apparatus.

Since it had been reasonably well established that vitamin C is an organic compound, or complex, of low molecular weight, it was evident that the amino acids would serve as a valuable basis of comparison for studying the function of the apparatus. In

TABLE I.

Concentrating Effect upon Amino Acids in Acid Solution, pH 0.9.

Sample.	Amino N found (Van Slyke method).			
	Glycine.		Phenylalanine.	
	Per 10 cc.	Total.	Per 10 cc.	Total.
	cc.	*cc.*	*cc.*	*cc.*
Cathode	7.4	61.0	8.9	60.5
Anode	2.3	18.9	3.3	25.7
Middle	4.3	298.8	6.4	473.6
Original	4.9	480.0	7.3	646.8

strongly acid solutions they function as cations due to their basic amino groups, and in alkaline solutions they function as anions, forming salts of their acid carboxyl groups. Glycine and phenylalanine were accordingly studied in filtered lemon juice to which had been added 20 cc. of concentrated hydrochloric acid per 980 cc. of juice. Electrolysis was continued for 5 hours at 1.5 amperes. Table I illustrates the results of a typical experiment.

It is evident from Table I that the amino acids were approximately 3 times more concentrated in the cathode chamber than in the anode chamber at the end of the run. It is also evident that the total loss of amino acids into the wash water around the electrode coils was comparatively small.

Table II shows the results obtained when electrolysis was con-

tinued for 5 hours with a current of 1.0 ampere in a strongly acid solution.

The concentrating effect is again seen to be approximately 3:1 between cathode and anode chambers, with a comparatively small loss of amino nitrogen in the electrode wash.

A slightly basic solution of lemon juice (pH 7.0 to 7.2) was then prepared by the addition of ammonia, and the transfer of amino acids was again studied. The results are shown in Table III.

In the above experiment the concentration in the anode chamber

TABLE II.

Concentrating Effect upon Glycine in Acid Solution, 1.0 Ampere for 5 Hours.

Sample.	Amino N from 10 cc.	Total volume.	Amino N in total volume.	pH.
	cc.	*cc.*	*cc.*	
Cathode	6.6	60	39.6	1.45
Anode	2.2	60	13.2	2.21
Middle	6.1	795	484.9	1.62
Cathode wash	0.95	300	28.5	12.3

TABLE III.

Concentrating Effect upon Leucine in Slightly Alkaline Solution, pH 7.0 to 7.2.

Sample.	Amino N per 10 cc.	Amino N in total volume.	pH.
	cc.	*cc.*	
Cathode	1.75	14.0	7.2
Anode	2.75	22.0	7.1
Middle	2.25	157.5	7.0
Original	2.40	227.8	7.0

is seen to be approximately 1.6:1 compared to the cathode chamber. A comparatively lower concentrating effect would be expected because the alkalinity used was much nearer the isoelectric point than were the acid solutions.

Solutions of lemon juice were electrolyzed under the same conditions used for the amino acids, and the resulting solutions from the three chambers were fed to guinea pigs, whose general condition, weight changes, and autopsy records, furnished evidence of the concentrations of the active factor in the three compartments

of the cell. A quantitative scoring of bony structure and hemorrhages was made, indicating the degree of scurvy. The scoring system and basal diet were substantially that of Sherman, La Mer, and Campbell (3). The electrolytic preparations were made every 3 days and kept under nitrogen in an ice box for daily feeding from graduated pipettes.

The results obtained are shown in Tables IV to VI.

In this experiment a cell with porous collodion bags for the anode and cathode chambers was used, and dilute citric acid was used for the electrode chamber electrolyte at the beginning of the run.

TABLE IV.

Normal Lemon Juice, pH 2.4.

Sample.	Amount of preparation fed daily.	No. of animals.	Average gain.	Average scurvy score.	Average survival (60 day test).
	cc.		*gm.*		
Cathode	1.5	2	196	0	60
	3.0	2	255	0	60
Middle	1.5	2	208	0	60
	3.0	3	166	0	60
Anode	1.5	2	164	0	60
	3.0	3	172	0	60
Negative controls	0	2	−93	18	29

Since, as shown in Table IV, 1.5 cc. of lemon juice after electrolysis were found to be sufficient to prevent scurvy and 3 cc. much above that amount, it was decided to make the dosage 1 and 2 cc. in further experiments. A slight increase or decrease in vitamin C concentration is easily noted with such doses because 1 cc. is just below the minimum protective dose and 2 cc. slightly above. Table V shows that there was no marked concentrating effect upon the vitamin and no marked loss from any electrode chamber, since all three chambers retained nearly their original activity.

The close similarity of the solutions from each chamber, as

given in Table IV, all of which correspond to untreated lemon juice, shows that there was no appreciable electrical transference

TABLE V.

Hyperacid Solution of Lemon Juice, pH 0.9.

20 cc. of concentrated HCl added to 980 cc. of lemon juice.

Sample.	Amount of preparation fed daily.	No. of animals.	Average gain.	Average scurvy score.	Average survival (56 day test).
	cc.		*gm.*		
Cathode..............	1	5	60	4	56
	2	5	82	1	56
Middle...............	1	5	97	1	56
	2	5	169	0	56
Anode................	1	5	88	2	56
	2	5	153	0	56
Negative controls.....	0	2	−78	17	25

TABLE VI.

Basic Solution of Lemon Juice, pH 7.0 to 7.2.

3.2 to 3.5 cc. of concentrated ammonia solution added to 900 cc. of lemon juice.

Sample.	Amount of preparation fed daily.	No. of animals.	Average gain.	Average scurvy score.	Average survival (41 day test).
	cc.		*gm.*		
Cathode..............	1	4	−14	8	41
	2	4	17	2	41
Middle...............	1	3	99	2	41
	2	4	134	0	41
Anode................	1	4	124	0	41
	2	3	148	0	41
Negative controls....	0	2	−81	19	24

of the vitamin at the pH of natural lemon juice. Full protection on 1.5 cc. also shows that vitamin C diffused readily through the

porous collodion membranes. This is of interest in view of a recent paper by Zilva (4) in which previous evidence regarding diffusibility was considered open to question.

The results in a hyperacid solution (Table V), show definitely that there was no marked transfer towards the cathode such as would be obtained if the active material were a basic substance or an ampholyte such as an amino acid. The middle and anode chambers were almost identical and equal to natural lemon juice. The small difference shown favors a higher concentration in the anode chamber than in the cathode chamber.

In the basic solution, Table VI, there is a distinct difference in activity of the solutions from the three chambers, indicating a transference towards the anode, such as would characterize a weak acid. A great many types of organic compounds might show such acidic properties, however, so that this evidence does not conflict with previous evidence that the active substance does not readily yield a volatile ester (5). This conclusion is also in harmony with the fact that it is precipitated by lead salts from basic solution and not from acid solution, and is not readily precipitated from solution as a barium salt (6). It is also of interest in relation to the close association of vitamin C with the actively reducing uronic acid isolated by Szent-Gyorgyi (7).

SUMMARY.

A new type of electrical transference apparatus, which protects the solution from alkali, oxygen, chlorine, and heat liberated during electrolysis, as well as oxygen from the air, has been devised and used for a study of the acid-basic properties of vitamin C. The activity of lemon juice in the anode, cathode, and middle compartments after 5 hours electrolysis was measured for antiscorbutic value by feeding to guinea pigs. Evidence was found for the rapid diffusibility of vitamin C through collodion membranes. Comparative measurements were made for the transfer of amino acids in acid and faintly alkaline solutions, showing a marked concentration in the cathode chamber in acid solution and in the anode chamber in alkaline solution (pH 7.2). Vitamin C showed no distinct transference towards the cathode in strongly acid solution (pH 0.9) nor in natural lemon juice (pH 2.4). It is concluded that the antiscorbutic vitamin is not a salt-forming nitrogen compound

such as an amino acid. In slightly alkaline solution there was evidence of a concentrating effect in the anode chamber, indicating that the active substance is acidic in nature. These findings are in harmony with previous evidence concerning the chemical nature of vitamin C.

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STUDIES ON CRYSTALLINE UREASE.

INACTIVATION BY ULTRA-VIOLET RADIATION, SUNLIGHT WITH THE AID OF A PHOTODYNAMIC AGENT, AND INACTIVATION BY TRYPSIN.

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The effect of sunlight and ultra-violet rays upon crystalline urease with the aid of eosin as a sensitizing agent, and also the effect of trypsin was the subject of our study.

Crystalline urease was prepared, after Sumner (1), from jack bean meal.[1] The crystals from 100 gm. of jack bean meal were dissolved in 25 cc. of distilled water at room temperature by stirring, and centrifuged free from insoluble matter. The watery solution was diluted with 25 cc. of a 2 per cent neutralized gum arabic solution, and was brought to pH 7. The solution, thus prepared, keeps well and was used for the experiments.

The activity of the jack bean meal was found to be 135 units and the activity of the crystals 105.000 units per gm. of dry material, as determined by the methods of Sumner (1) and Sumner and Hand (2).

The degree of inactivation was determined by drawing off small samples of the urease solution at the time intervals stated below; the ammonia formed was determined by aeration and direct Nesslerization.

A 0.01 per cent solution of eosin Y, yellowish[2] (alcohol- and water-soluble), in distilled water was used for inactivation. All solutions were preserved with toluene, and the experiments were performed under sterile conditions.

[1] Eimer and Amend, No. B 30.

[2] Coleman and Bell Company.

Sunlight and Eosin.

The experiments were started on April 15 at 12.15 p.m. The temperature was 23°. Small white porcelain crucibles, 70 mm. in their largest diameter and 45 mm. deep, were used.

As a control 5 cc. of the urease solution were exposed to sunlight without the addition of eosin for 30 minutes. No loss of activity was noted. To 5 cc. of urease was added 0.05 cc. of eosin, and the solution was put into a water bath at 23°. No loss of activity was observed. 5 cc. samples of urease, to which was added 0.05 cc. of 0.01 per cent eosin, were exposed to sunlight at 23° for varying lengths of time. The results are shown below. The addition of eosin causes a complete inactivation of the enzyme within 20 minutes.

Sample No.	Time.	Per cent present.
	min.	
1	5	76
2	10	52
3	15	10
4	20	0

Ultra-Violet Rays and Eosin.

For these experiments a Hanovia quartz mercury vapor lamp was used, which was operated at 110 volts and 4 amperes. The results of some of the experiments are shown in Chart I.

If the urease is exposed to ultra-violet rays at a distance of 25 cm. with the addition of eosin, a complete inactivation results within 5 minutes. Exposure to the rays without eosin shows similarly, a decline in its activity, which, however, is more gradual and is only complete after 30 minutes. If the distance of the exposure is increased to 50 cm. without the addition of eosin, the process of inactivation is much slower, after 30 minutes 55 per cent of its activity still being present. The presence of eosin increases the rate of inactivation. After 30 minutes only 20 per cent of its activity is present.

We performed similar experiments with impure urease and found it more resistant than crystalline urease.

Inactivation by Trypsin.

In order to find out whether the activity of the crystalline urease is affected by proteolytic substances in the presence of a protective colloid, trypsin was added to the urease solution.

1 gm. of trypsin was dissolved in 90 cc. of a buffer of pH 7 to which 10 cc. of glycerol were added.

5 cc. of the urease-gum arabic solution were added to 5 cc. of trypsin-glycerol buffer solution and kept in an incubator at 37°. After 1 day, 55 per cent of the activity of the urease was destroyed;

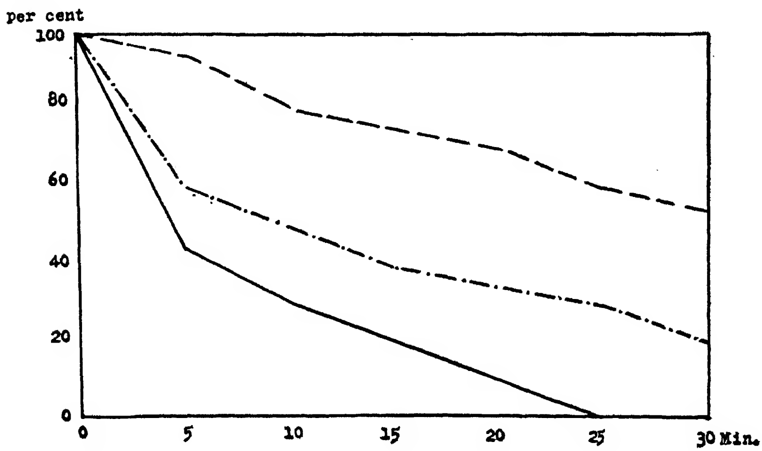

CHART I. Result of exposure of urease solutions to ultra-violet rays. ——— represents 5 cc. of urease, without eosin, at 41° exposed at 25 cm. — — —, 5 cc. of urease, without eosin, at 30° exposed at 50 cm. — · — · —, the same plus 0.05 cc. of 0.01 per cent eosin.

after 2 days, 90 per cent of its activity was lost; after 3 days the inactivation was complete. As a control, a sample of the crystalline urease-gum arabic solution was kept in the incubator at 37° for 3 days without any loss of activity.

SUMMARY.

1. Direct sunlight does not affect urease to which no eosin has been added at the various temperatures and time intervals. In the presence of eosin, sunlight inactivates the urease.

2. Ultra-violet rays have an inhibitory effect upon the activity of urease, which is inversely proportional to the distance. The presence of eosin increases this effect.

3. Crystalline urease is inactivated by trypsin in the presence of a protective colloid, which would indicate its protein nature.

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STUDIES IN THE PHYSICAL CHEMISTRY OF THE PROTEINS.

VII. THE SOLUBILITY OF FIBRINOGEN IN CONCENTRATED SALT SOLUTIONS.

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In 1859, Denis (8) observed the appearance of a gelatinous precipitate which he called plasmine in blood saturated with respect to sodium chloride.[1] Plasmine was studied by Leon Fredericq (11) in 1877. He noted that upon heat coagulation, proteins separated at two different temperatures and, therefore, suggested that plasmine consisted of two proteins, one of them the postulated protein in the blood concerned with its coagulation and called fibrinogen by Virchow. In 1879 Hammarsten (14) precipitated one of these proteins from blood plasma by half saturating it with respect to sodium chloride. This method has ever since been the classical procedure in the preparation of fibrinogen.

Fibrinogen has generally been considered insoluble in water, but soluble in dilute salt solutions and, therefore, to belong to that class of substances, the globulins, which were first described by Denis (8) in the same monograph in which he described plasmine. The contention of De Waele (9) that fibrinogen is largely soluble in water involves both the characterization of this protein and the concept of solubility, and these we shall consider in terms of the measurements that are reported.

The early observations upon the temperature of heat coagula-

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[1] The blood was rendered incoagulable by the addition of one-sixth of its volume of saturated ammonium sulfate.

tion have since been supplemented, but in this protein, as in others, the coagulation temperature varies with the concentration and the nature of the solvent. A method of characterization which has generally proved satisfactory is the determination of the isoelectric point. Various investigators have, however, reported values for the isoelectric point of this globulin, varying from pH 4.4 to 8. In Table I are collected their observations, together with the methods that have been employed in the attempts to measure the isoelectric point of fibrinogen.

TABLE I.

Measurements of the Isoelectric Point of Fibrinogen.

Author.	Year.	Method employed	Isoelectric pH.
Resch (23).	1917	Cataphoresis in phosphate buffer.	5.0–8.0
Funck (12).	1921	Maximal flocculation in acetate buffer.	4.4–5.0
Stuber and Funck (28).	1921	Cataphoresis in acetate buffer.	5.0
Kugelmass (17).	1923	Maximal turbidity.	8.0
Wöhlish (30).	1924	" flocculation in acetate buffer.	4.9
Rabinovich (22).	1925	Maximal opalescence.	5.9–6.2
Quagliariello (21).	1926	" flocculation in acetate buffer.	4.9
De Waele (9).	1927	Cataphoresis and maximal precipitation by alcohol.	6.9
Wöhlish and Schloss (31).	1927	Maximal flocculation in NaCl.	4.46
Nordbö (19).	1927	Titration curve.	5.50

Globulins are rendered more soluble by low concentrations, but less soluble by high concentrations of neutral salts. The latter property has most often been taken advantage of in the preparation of globulins, and the observation of Hammarsten (14) that fibrinogen was precipitated by half saturation with sodium chloride indicates that this is the most readily precipitated of the blood proteins. Since the preparation of fibrinogen thus depends upon its solubility in concentrated salt solutions, and since the precipitation of proteins by neutral salts has recently achieved not only a

quantitative formulation but a theoretical significance, both for the characterization of proteins and for the interpretation of the effects of different salts upon them, it seemed desirable to begin our studies upon this protein by determining its solubility in concentrated salt solutions.

Several investigators have determined the concentration of a neutral salt at which the precipitation of the protein begins. Data of this kind have been compiled by Pickering (20) and by Davide (6). They give us information only concerning the protein concentration of the actual salt solution considered, for precipitation starts when the protein is in greater concentration than the amount corresponding to saturation. The so called salting out of proteins by neutral salts has been quantitatively investigated in recent years. Chick and Martin (1), and later Sørensen and Høyrup (26), studied the precipitation of egg albumin by ammonium sulfate and noted the influence of the concentration of the neutral salt, of the hydrogen ion, and the apparent influence of the amount of saturating body. Sørensen and Høyrup (26) interpreted the apparent influence of the saturating body in terms of the water and the salt contained in the precipitate. They showed that, at any one temperature, the solubility of egg albumin was completely defined by the concentration of neutral salt and of hydrogen ions in the solution. Disturbances due to salt and water content of the precipitate, factors for which Sørensen and Høyrup corrected their results, become less significant if smaller amounts of saturating body are employed.

In 1925 Cohn (3) demonstrated that a linear relationship existed between the concentration of the added salt and the logarithm of the solubility. In sufficiently concentrated solutions solubility could, therefore, be described by the equation

$$\log S = \beta - Ks\mu \qquad (1)$$

He interpreted the constant β as representing "the solubility in water of the soluble protein compound in the absence of salt," ((3) p. 417) which is precipitated by the neutral salt. The proportionality constant he associated with the salting out constant employed by Debye and McAulay (7) in describing the effect of neutral salts upon the activity coefficients of non-electrolytes.

More recently Cohn and Prentiss (2) showed that the solvent action of neutral salts upon the globulin, hemoglobin, could be described by means of the first term of the Debye-Hückel equation for the activity coefficients of electrolytes

$$\log \frac{S}{S_0} = 0.5\, z_1 z_2 \frac{\sqrt{\mu}}{1 + \kappa b} - Ks\mu \qquad (2)$$

The last term in this equation may be associated with the last term in Equation 1 and the two equations considered identical under such circumstances that the first term is equal to zero, or assumes a constant value. Green and Cohn (13) have described the solubility of carboxyhemoglobin in solutions of sodium chloride and potassium chloride up to very concentrated solutions. Under these circumstances it became apparent that there was a salting out effect, and the value of the salting out constant, Ks, was calculated. Neither sodium chloride nor potassium chloride can, however, be regarded as precipitants of carboxyhemoglobin. Carboxyhemoglobin is, however, completely precipitated by the phosphates or sulfates of sodium, ammonium, or potassium.

Cohn and Green (5) have studied the solubility of carboxyhemoglobin in solutions of these salts at different temperatures and acidities and have shown that, in sufficiently concentrated solutions, solubility is described, within the accuracy of their measurements, by Equation 1. Presumably, when the concentration of the solvent is sufficiently great, the first term on the right-hand side of Equation 2 either approaches a constant value or becomes negligible in comparison to the last term, and, under these circumstances, the simpler equation first employed in describing the properties of albumins suffices for the characterization of globulins. This investigation upon carboxyhemoglobin appears to confirm the deduction drawn from the study of the albumins that the salting out constant, Ks, is characteristic of a given protein and a given salt, and independent of pH. They also showed that it was independent of temperature. On the other hand, β is a variable describing the amphoteric behavior of the protein.

In the present investigation we have determined the values of Ks and β for fibrinogen in different salt solutions and at different acidities, and, as a result, have reduced to quantitative terms a

description both of those properties of the protein that depend upon its acid and basic dissociation, and of those upon which depends its precipitation by neutral salts.

Preparation of Fibrinogen.[2]

The method devised by Hammarsten (14) for the separation of fibrinogen depended upon its precipitation by the addition to plasma of an equal amount of a saturated solution of sodium chloride. Purification was achieved by repeatedly dissolving fibrinogen in dilute sodium chloride and precipitating it by half saturating the solution with respect to the salt. Heubner (16) showed that after precipitation at an alkaline reaction the resulting product was composed only to a small extent of fibrinogen, but to a much greater extent of a substance having very different properties. Only when purification was carried out at a neutral reaction did his product consist largely of fibrinogen. The following method of preparing fibrinogen, which depends upon the observations of Hammarsten and Heubner, was therefore attempted.

Method 1.—Citrated horse plasma, after remaining in a cold room for 1 day, was filtered, neutralized to litmus with dilute acetic acid, and rendered half saturated with respect to sodium chloride. The precipitation was carried out at room temperature, the neutral and calcium-free salt being slowly added. At least an hour was allowed to elapse after all the salt solution had been added before the precipitate was removed from the plasma by centrifugation. The precipitate was redissolved in an approximately normal sodium chloride solution (5 per cent) with the aid of gentle mechanical agitation. This process was thrice repeated, the solution remaining in the cold room only between the successive precipitations.

It was shown by Nolf (18) that preparations obtained by Hammarsten's method at room temperature were unsuitable for experiments upon the transformation of fibrinogen into fibrin. Presumably changes in the nature of the protein occurred under these circumstances which modified its reactions. Nolf, therefore, carried out his preparations in vessels cooled to 0°. These pre-

[2] We are indebted to the Massachusetts Antitoxin Laboratory for their courtesy in supplying us with the plasma from which fibrinogen was prepared.

cautions we observed in a second method of preparing fibrinogen, analogous to the first one, and differing only in this: that from the time the plasma was separated from the cells, the solutions were never taken from a cold room, maintained at $2° \pm 0.2°$. The following experiments indicate the necessity for this procedure, always subsequently employed.

Throughout this investigation the criteria of solubility were employed in determining whether a single protein had been isolated from the plasma. When the preparation had been carried out essentially as described in Method 1, solubility was studied in a phosphate buffer solution consisting of 0.49 mol of KH_2PO_4 and 0.53 of K_2HPO_4 and, therefore, having an ionic strength of 2.09 and a pH of 6.6 (4). The solubility of such a preparation did not remain constant, independent of the amount of saturating body. The results are recorded in Table II, in which it will be seen that, far from being constant, solubility increased progressively as the protein precipitate was successively triturated with fresh portions of the phosphate buffer.

When the precaution of carrying out the entire preparation at a low temperature was scrupulously observed, however, fibrinogen of constant solubility was prepared. The results of a single experiment are given in Table III, in which it will be observed that fibrinogen dissolved in the successive amounts of phosphate buffer with which it was equilibrated, to the same extent, that saturation was complete when 0.017 gm. of fibrinogen nitrogen per liter of the phosphate buffer had dissolved, that 2 hours appeared to be a sufficiently long time for saturation, and that more prolonged equilibration did not influence solubility. This protein must, therefore, be considered to have been separated from the other proteins of the plasma and to have been prepared in a state of purity with respect to other proteins and to the products of its own decomposition.

The difference in behavior of fibrinogen prepared by Methods 1 and 2 explains, we believe, statements such as those made by Wöhlish (30) that successive reprecipitations make fibrinogen more and more unstable. An examination of the methods used by the different authors quoted in Table I suggests that the fibrinogen solutions on which they made isoelectric determinations were prepared by methods which probably yielded a mixture of fibrinogen and its decomposition products.

If a solution, prepared by Method 2 and completely precipitable by half saturation with sodium chloride, is dialyzed in a collodion

TABLE II.

Experiment 5. Fibrinogen Preparation 14.

Phosphate ionic strength = 2.09, pH = 6.6, temperature = 25° ± 0.2°.

Addition of phosphate buffer to precipitate.	Time precipitate was equilibrated with phosphate buffer.	Solubility.
	hrs.	*gm. N per l.*
1st	1	0.035
	15	0.080
2nd	1	0.085
	2	0.090
	3	0.115
	6	0.262
3rd	6	0.575

December 3, 1928. To 2 liters of fibrinogen solution prepared by Method 1 were added 2 liters of a phosphate buffer consisting of 1 mol of KH_2PO_4 and 1 mol of K_2HPO_4. The precipitate was redissolved in 0.1 molal phosphate of similar composition, and reprecipitated by means of an equal volume of the 2 molal phosphate buffer.

The precipitate, collected by centrifugation, was placed in a 200 cc. flask. 50 cc. of a precisely 1.02 molal phosphate buffer (mol fraction K_2HPO_4 = 0.52), ionic strength 2.09, pH 6.6, were added. A few drops of toluene were also added to prevent bacterial action. The flask was put on a shaking machine in a water bath kept at 25° ± 0.2°. Samples were collected after different periods of shaking, filtered, and analyzed with respect to nitrogen. Fresh solution was added to the precipitate and the process repeated two more times. As shown by the figures in Table II, no equilibrium was attained, the solubility increasing with time. At the end of the experiment the remaining precipitate was entirely soluble in dilute phosphate. The protein dissolved during the third equilibration with the precipitate was, however, no longer completely precipitated upon the addition of an equal volume of a saturated sodium chloride solution. 4 volumes of saturated sodium chloride precipitated all of the coagulable protein present, but the filtrate from this precipitate still contained 0.020 mg. of nitrogen per cc.

membrane in the cold, under reduced pressure, and in the presence of toluene, against an alkaline solution, by the time the fibrinogen has become salt-free it has also become denatured. The addition

of an equal volume of saturated sodium chloride no longer yields a precipitate even after neutralization of any residual alkali. Since fibrinogen is characterized and prepared by virtue of its insolubility under these circumstances, the conclusion is inevitable that hydrolysis of the fibrinogen had proceeded during the dialysis, yielding a product of different properties than fibrinogen. In order to

TABLE III.

Fibrinogen Preparation 18.

Phosphate ionic strength = 2.09, pH = 6.6, temperature = 25° ± 0.2°.

	Addition of phosphate buffer to precipitate.	Time precipitate was equilibrated with phosphate buffer.	Solubility.	Fibrinogen.
		hrs.	*gm. N per l.*	*gm. per 1000 gm. H_2O*
Experiment 9.	1st	2	0.017	0.106
	2nd	2	0.017	0.106
	3rd	4	0.018	0.110
	4th	11	0.017	0.106
Experiment 10.	1st	2	0.017	0.106

December 20, 1928. To 1 liter of fibrinogen solution prepared by Method 2 was added 1 liter of phosphate buffer consisting of 1 mol of KH_2PO_4 and 1 mol of K_2HPO_4. The precipitate was redissolved in a 0.1 molal phosphate buffer of similar composition, reprecipitated, and redissolved, and again reprecipitated with the 2 molal phosphate buffer.

The precipitate, collected by centrifugation, was put in a 200 cc. bottle. 100 cc. of a precisely 1.02 molal buffer (mol fraction K_2HPO_4 = 0.524, μ = 2.09, pH = 6.6) were added after washing with the same solution. The bottle was placed at 25° ± 0.2°. Samples were collected after a period of shaking, filtered, and analyzed with respect to nitrogen. Fresh solution was added to the precipitate and the process repeated three more times. At the end of the experiment, the precipitate was still completely soluble in dilute phosphate.

determine whether the hydrolysis of the fibrinogen during dialysis was dependent on maintaining an alkaline reaction we dialyzed, in two membranes, a fibrinogen solution prepared by Method 2 and completely precipitable by half saturation with sodium chloride. One membrane was dialyzed against the alkaline solution recommended by Heubner (16), an approximately 0.0007 molal sodium hydroxide solution, the other against distilled water at pH 6.

Even after 1 day the solution dialyzed against dilute alkali was only partially precipitable by half saturation with sodium chloride. That a modification of the fibrinogen had occurred, yielding a less readily precipitable product, was demonstrated by heat coagulation of the filtrate from a half saturated sodium chloride solution.

The fibrinogen dialyzed at a neutral reaction soon began to precipitate. This precipitate was relatively insoluble in water but completely soluble in 5 per cent sodium chloride solution, from which it could be completely precipitated by half saturation with sodium chloride. This product, therefore, still manifested the characteristics of fibrinogen[3] and led to the method of preparation that was finally adopted. This may be described as follows:

Method 3.—Citrated horse plasma is kept in the cold room for 1 day, filtered, and the reaction brought to about pH 6.0 by means of dilute hydrochloric acid. An equal volume of a saturated calcium-free solution of sodium chloride of pH 6.0 is slowly added through a glass tube extending below the surface of the plasma and close to a screw-shaped motor-driven glass stirrer which constantly brings new portions of plasma in contact with the salt solution and avoids the accumulation of a local excess of reagent. The precipitate is allowed to collect for 1 hour, and is then filtered. It is redissolved by stirring in 5 per cent sodium chloride at pH 6.0. The solution is filtered and toluene added. The process is thrice repeated. The solutions are never taken out of the cold room, and all the operations are performed there.

In order to reduce denaturation of fibrinogen as far as possible we have always maintained our solutions in the cold at a reaction near pH 6 and tested their complete precipitability in a half saturated solution of sodium chloride before and after each experiment.

The solutions prepared by this method are slightly opalescent, are completely precipitated on half saturation with sodium chloride, and when kept in the cold room with the addition of a few drops of

[3] Dialyzed fibrinogen deteriorates to some extent even under the best experimental conditions that we have thus far been able to devise. Fibrinogen precipitated by dialysis and kept suspended in distilled water saturated with toluene appears to change slowly and in two respects: on the one hand it loses its solubility in sodium chloride; on the other, that portion which remains soluble is no longer completely precipitable by half saturation with sodium chloride.

toluene, remain unchanged for weeks. They are coagulated by the addition of horse serum.

Measurement of Solubility.

In order that solubility measurements may have a theoretical significance they must be so carried out that solubility is independent of the amount of saturating body and of the time of equilibration. The results recorded in Table III demonstrated that it was possible to prepare fibrinogen sufficiently pure to satisfy these conditions at least within the limits of error obtained in these measurements. The temperature has been maintained constant at 25° ± 0.2° in all of these experiments, and the pH of each solution was always measured.

Certain modifications of the procedures that have generally been employed in measuring solubility in this laboratory were imposed by the properties of fibrinogen that have already been described. For the most part it was found desirable to reach equilibrium by precipitation of a fibrinogen solution rather than by dissolving precipitated fibrinogen. That equilibrium was reached from either side is indicated by the experiments recorded in Table III. In these experiments fibrinogen was precipitated by the phosphate buffer, was centrifuged, and the supernatant liquid decanted. The precipitate was then partially dissolved by being triturated with the more dilute phosphate buffer solution indicated, and solubility was measured. That this procedure gives the same result as that which has generally been employed may be seen by comparing this result with those in Table VI. The measurements recorded in Table III are also graphically represented in Fig. 1 where they fall upon the straight line which described solubility in phosphate buffers of this pH.

Sørensen and Høyrup (27) have demonstrated that equilibrium is reached more rapidly in the case of egg albumin when crystals are dissolved than when crystallization is brought about by increase in the concentration of neutral salt, and this phenomenon appears to be of general occurrence. Experiments were undertaken to determine the length of time required for precipitation to be completed, within the accuracy of our measurements, of a fibrinogen solution that had been added to a concentrated salt solution.

The procedure that has generally been followed has been to

reprecipitate the fibrinogen sufficiently often with the salt in the solution in which its solubility was to be determined, so that no other ions were present. The reaction of fibrinogen in this neutral salt solution was adjusted to the desired pH by means of acid or alkali. Aliquot parts were then placed in bottles in a water bath at 25° and permitted to come to this temperature. A water-driven screw-shaped glass stirrer dipped into each bottle and after temperature equilibrium was reached the solutions were stirred and the desired amounts of saturated salt solution slowly added from a pipette, the tip of which reached below the surface of the liquid and just above the stirrer which constantly forced fresh portions of the solution past. After the salt solutions were added, stirring was continued for the length of time that had been found necessary for equilibrium. The solutions were then filtered on No. 42 Whatman filter papers. The temperature of the filtration was maintained as close as possible to that of the bath by the use of water-jacketed filter funnels. Solubility was always estimated by analyzing the filtrate with respect to nitrogen by the Kjeldahl method. The concentration of neutral salt in the filtrates was analyzed by methods appropriate for the specific ions. Hammarsten (15) estimated that fibrinogen contained 16.66 per cent of nitrogen. The nitrogen analyses made on the various saturated fibrinogen solutions have, therefore, been multiplied by 6 to yield fibrinogen concentrations.

Solubility in Sodium Chloride Solutions.

Since the preparation of fibrinogen has, in the past, depended largely upon its precipitation by sodium chloride, the solubility of this protein in solutions of this neutral salt was quantitatively investigated. The fibrinogen for these studies was prepared as a solution in 5 per cent sodium chloride. The solubility experiments were carried out in the manner that has been described, the concentration of sodium chloride in the saturated solutions being estimated by chloride analysis carried out by Wilson's modification (29) of Van Slyke's method. The pH was measured electrometrically. The results obtained at pH 5.8 are recorded in Table IV and are defined by the equation

$$\log S = 1.61 - 0.91\mu$$

in which 0.91 is the value for Ks, and 1.61 for β.

Solubility in Potassium Phosphate Solutions.

From certain points of view phosphate buffer solutions must be considered preferable for the extraction and the precipitation of proteins to most other salts. This is especially true where small changes in reaction are likely to denature a protein, or, from an analytical point of view, where solubility is changing rapidly with change in pH. The determination of the activity coefficients and,

TABLE IV.

Solubility of Fibrinogen in Concentrated Solutions of Sodium Chloride. Temperature = 25° ± 0.2°, pH = 5.8. Defined by the equation $\log S = \beta - K_s\mu$.

	Concentration of total protein.	Period of stirring.	Sodium chloride concentration.		Ionic strength.	Solubility of fibrinogen.		Log of solubility.	$\frac{\text{Log } S_1 - \log S_2}{\mu_1 - \mu_2}$	$\text{Log } S + 0.91\mu$
	gm. per l.	*hrs.*	*M per l. solution*	*M per 1000 gm. H_2O*	μ	*s = gm. per l. solution*	*S = gm. per 1000 gm. H_2O*	*log S*	K_s	β
Experiment 20, Preparation 29.	1.360	1	2.35	2.46	2.46	0.230	0.241	$\bar{1}.38$	0.91	1.61
	1.310	1	2.43	2.55	2.55	0.192	0.206	$\bar{1}.31$	0.93	1.61
	1.270	1	2.49	2.62	2.62	0.168	0.176	$\bar{1}.24$	0.89	1.62
	1.230	1	2.61	2.75	2.75	0.132	0.139	$\bar{1}.14$	0.90	1.64
	1.220	1	2.64	2.78	2.78	0.120	0.126	$\bar{1}.10$	0.86	1.62
Experiment 25, Preparation 36.	0.980	1	2.04	2.12	2.12	0.540	0.562	$\bar{1}.74$	1.02	1.66
	0.980	3	2.04	2.12	2.12	0.480	0.500	$\bar{1}.69$	0.87	1.61
	0.945	3	2.18	2.27	2.27	0.378	0.394	$\bar{1}.59$	0.97	1.65
	0.810	3	2.28	2.38	2.38	0.264	0.276	$\bar{1}.44$	0.92	1.60
	0.820	3	2.55	2.68	2.68	0.132	0.139	$\bar{1}.14$	0.91	1.57
Average									0.91	1.61

therefore, of the apparent dissociation constant of mixtures of KH_2PO_4 and K_2HPO_4 has facilitated the preparation of phosphate buffers of known pH and ionic strength, and it is believed that for purposes of the characterization of proteins, solubility in such solutions will prove most valuable.

Fibrinogen preparations employed in these measurements were first purified of other proteins by precipitation with sodium chloride by the method that has already been described (Method 3).

Finally the fibrinogen was purified of sodium chloride by being twice reprecipitated by a 2 molal phosphate buffer (mol fraction $K_2HPO_4 = 0.52$).

TABLE V.

Solubility of Fibrinogen in Concentrated Phosphate Solutions. Fibrinogen Preparation 26.

Mol fraction $K_2HPO_4 = 0.52$, temperature = 25° ± 0.2°, pH = 6.6. Concentration of total protein in the system = 2.80 gm. per liter; concentration of phosphate = 0.82 mol per liter.

Time of equilibration.	Solubility.
hrs.	*gm. fibrinogen per l.*
1	2.586
2	2.580

TABLE VI.

Solubility of Fibrinogen in Concentrated Solutions of $KH_2PO_4 + K_2HPO_4$.

Mol fraction $K_2HPO_4 = 0.52$, pH = 6.6, temperature = 25° ± 0.2°, period of stirring = 1 hour. Defined by the equation $\log S = \beta - Ks\mu$.

	Concentration of total protein.	Phosphate concentration.		Ionic strength.	Solubility of fibrinogen.		Log of solubility.	$\frac{\text{Log } S_1 - \log S_2}{\mu_1 - \mu_2}$.	Log S + 1.95μ.
	gm. per l.	*M per l. solution*	*M per 1000 gm. H_2O*	μ	s = *gm. per l. solution*	S = *gm. per 1000 gm. H_2O*	*log S*	*Ks*	β
Experiment 15, Preparation 26.	3.040	0.712	0.736	1.49	1.650	1.706	0.24	1.92	3.14
	2.800	0.820	0.852	1.73	0.522	0.542	$\bar{1}.73$	1.93	3.16
	2.690	0.867	0.902	1.84	0.288	0.299	$\bar{1}.47$	1.97	3.05
	2.560	0.919	0.958	1.95	0.186	0.194	$\bar{1}.28$	2.05	3.08
Experiment 16, Preparation 26.	3.170	0.664	0.685	1.39	2.580	2.664	0.42	1.94	3.13
	3.040	0.719	0.744	1.51	1.680	1.739	0.23	2.02	3.17
	2.920	0.775	0.802	1.64	0.780	0.809	$\bar{1}.90$	2.12	3.09
	2.840	0.809	0.841	1.71	0.540	0.561	$\bar{1}.74$	1.78	3.07
Experiment 19, Preparation 28.	0.752	1.021	1.071	2.18	0.072	0.075	$\bar{2}.87$	1.81	3.12
	0.752	0.973	1.017	2.09	0.093	0.097	$\bar{2}.98$	2.00	3.05
	0.752	0.925	0.976	1.98	0.162	0.169	$\bar{1}.22$	1.98	3.08
	0.752	0.877	0.914	1.86	0.300	0.312	$\bar{1}.49$	1.93	3.11
	0.752	0.829	0.861	1.76	0.450	0.467	$\bar{1}.66$	2.02	3.09
Average.								1.95	3.09

Solubility measurements were carried out by means of the same procedure as was employed in the case of sodium chloride. A

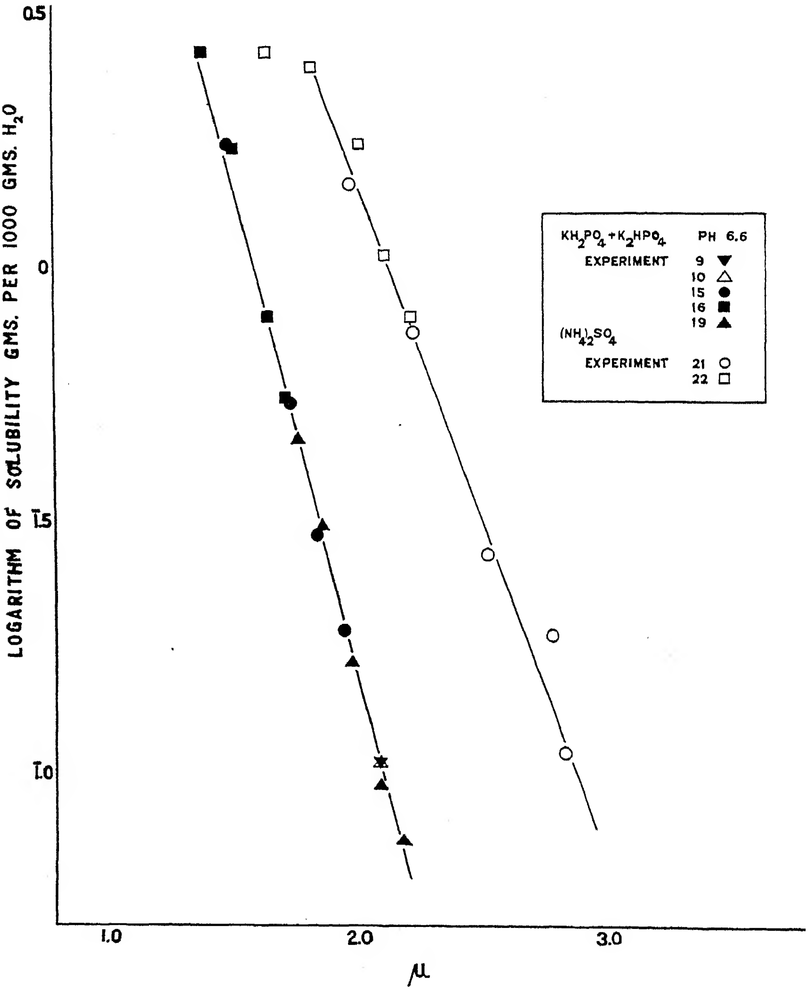

Fig. 1. Solubility of fibrinogen in concentrated phosphate and sulfate solutions at varying ionic strength.

preliminary experiment, recorded in Table V, suggested that equilibrium was reached within an hour. The results of a large

number of measurements all made at pH 6.6 are recorded in Table VI. The amount of potassium phosphate in the saturated fibrinogen solutions was estimated by analyzing the filtrates for phosphate by the method of Fiske and Subbarow (10).

The results in Tables III, V, and VI are graphically represented in Fig. 1. They leave little doubt that the logarithm of solubility is inversely proportional to the molecular concentration, or the ionic strength. This proportionality is represented by the equation

$$\log S = 3.09 - 1.95\mu$$

in which $\beta = 3.09$ and $Ks = 1.95$.

TABLE VII.

Solubility of Fibrinogen in Concentrated Ammonium Sulfate Solutions. Fibrinogen Preparation 32.

Temperature = 25° ± 0.2°. Concentration of total protein in the system = 2.91 gm. per liter; concentration of $(NH_4)_2SO_4$ = 0.562 mol per liter.

Time of equilibration.	Solubility.
hrs.	*gm. fibrinogen per l.*
1	2.586
2	2.580

Solubility in Ammonium Sulfate Solutions.

Ammonium sulfate has been the classical salt employed in the precipitation of proteins and their separation from each other. The fibrinogen preparations were first precipitated in sodium chloride in the conventional manner, and then twice precipitated and redissolved in ammonium sulfate solutions.

The presence of ammonia in the filtrate very much complicates the measurement of solubility in such solutions, however, and it is in part for this reason that phosphate buffers are superior for purposes of characterization.

The procedure employed in this investigation was based on that adopted by Sørensen and Høyrup (25) in their study on egg albumin. The fibrinogen in the filtrates was coagulated on a water bath, then cooled, and filtered. The filters on which the precipitates

TABLE VIII.

Solubility of Fibrinogen in Concentrated Solutions of Ammonium Sulfate.

Temperature = 25° ± 0.2°, pH = 6.0, period of stirring = 1 hour. Defined by the equation log $S = \beta - Ks\mu$.

	Ammonium sulfate concentration.		Ionic strength.	Solubility of fibrinogen.		Log of solubility	Log S + 1.33μ.
	M per l. solution	M per 1000 gm. H_2O	μ	s = gm. per l. solution	S = gm. per 1000 gm. H_2O	log S	β
Experiment 11, Preparation 20.	0.640	0.665	1.99	0.780	0.811	$\bar{1}.90$	2.54
	0.690	0.719	2.15	0.360	0.375	$\bar{1}.57$	2.42
	0.770	0.807	2.42	0.258	0.272	$\bar{1}.43$	2.64
	0.850	0.896	2.68	0.090	0.094	$\bar{2}.97$	2.53
	0.950	1.008	3.02	0.042	0.044	$\bar{2}.64$	2.45
Average							2.51

TABLE IX.

Solubility of Fibrinogen in Concentrated Solutions of Ammonium Sulfate.

Temperature = 25° ± 0.2°, pH = 6.6, period of stirring = 1 hour. Defined by the equation log $S = \beta - Ks\mu$.

	Concentration of total protein.	Ammonium sulfate concentration.		Ionic strength.	Solubility of fibrinogen.		Log of solubility.	$\frac{\text{Log } S_1 - \log S_2}{\mu_1 - \mu_2}$.	Log S + 1.33μ.
	gm. per l.	M per l. solution	M per 1000 gm. H_2O	μ	s = gm. per l. solution	S = gm. per 1000 gm. H_2O	log S	K_s	β
Experiment 21, Preparation 31.	2.65	0.633	0.657	1.97	1.416	1.471	0.16	1.30	2.78
	2.65	0.710	0.741	2.22	0.726	0.758	$\bar{1}.87$	1.42	2.82
	2.65	0.803	0.843	2.52	0.264	0.277	$\bar{1}.43$	1.29	2.78
	2.65	0.850	0.896	2.68	0.180	0.189	$\bar{1}.27$	1.29	2.83
	2.65	0.893	0.944	2.83	0.105	0.111	$\bar{1}.04$	1.41	2.80
Experiment 22, Preparation 32.	2.91	0.562	0.581	1.74	2.586	2.675	0.42	1.01	2.73
	2.91	0.588	0.609	1.82	2.418	2.507	0.39	1.21	2.81
	2.91	0.645	0.670	2.01	1.680	1.747	0.24	1.58	2.91
	2.91	0.678	0.706	2.11	1.032	1.075	0.02	1.36	2.82
	2.91	0.706	0.737	2.21	0.762	0.795	$\bar{1}.90$	1.50	2.83
Average								1.33	2.81

were deposited, were washed with hot water until free of sulfate. The precipitate was then considered to be free from ammonia nitrogen and was analyzed for fibrinogen nitrogen by the Kjeldahl method. The filtrates were collected in volumetric flasks, brought to known volumes with boiled distilled water, and the ammonium sulfate concentrations estimated by the determination of ammonia.

Solubility has been studied in ammonium sulfate solution at pH 6 and 6.6. 1 hour appeared to be sufficient for the establishment of equilibrium in this, as in the other salts investigated (Table VII). The results at pH 6 are represented in Table VIII and those at pH 6.6 in Table IX. In Fig. 1 is contrasted the solubility of fibrinogen at pH 6.6 in concentrated phosphate and sulfate solutions. There can be no doubt that the data are adequately represented by a straight line for the sulfates, as well as for the phosphates, although the technical difficulties in making the measurements are far greater in the case of ammonium sulfate. The equations defining solubility in ammonium sulfate are

$$\text{For pH 6.0, } \log S = 2.51 - 1.33\,\mu$$
$$\text{" " 6.6, " " } = 2.81 - 1.33\text{"}$$

Solubility of a Protein in a Salt Solution at Different Acidities.

Sørensen and Høyrup (26) studied the solubility of egg albumin at different acidities, and the recalculation of their results by Cohn (3) indicated that for the same salt solution Ks is independent of pH. Our results with fibrinogen are consistent with this generalization. Moreover, the studies upon egg albumin and carboxyhemoglobin indicate that solubility is minimal near the isoelectric point of a protein. That is to say, β has a minimal value in the case of egg albumin near pH 4.5 and for carboxyhemoglobin near 6.6. Their respective isoelectric points are 4.7 and 6.78. Since the solubility of fibrinogen is lower in ammonium sulfate at pH 6 than at pH 6.6, these data suggest that the isoelectric point of this protein occurs at a more acid reaction. These observations thus throw a certain light on the isoelectric measurements in Table I and favor the acceptance of an acid value for this constant.

Solubility of Different Proteins in the Same Salt Solutions.

The solubility of egg albumin, of serum albumin, of pseudoglobulin, of carboxyhemoglobin, and of fibrinogen have now been

studied in concentrated solutions of ammonium sulfate. The value of *Ks* varies in the proteins under investigation, as shown in Table X. The order is presumably a measure of the extent to which these proteins alter the dielectric properties of solutions. It does not necessarily yield the order in which these proteins will be precipitated from the same solution. A second set of influences is simultaneously operative. These influences are designated by β and ascribed to the amphoteric nature and state of the protein (3). A change in pH, which will not within wide limits affect the value of *Ks* changes the dissociation of a protein as acid and as base and,

TABLE X.

Values of β and Ks in the Equation Log $S = \beta - Ks\mu$, Defining Solubility of Different Proteins in Concentrated Solutions of Ammonium Sulfate.

Protein.	Author.	pH	*Ks**	β
Fibrinogen.		6.6	1.33	2.81
		6.0	1.33	2.51
Pseudoglobulin.	Sørensen (25).		1.00	5.44
Egg albumin.	Chick and Martin (1).		0.96	7.85
" "	Sørensen and Høyrup (27).	4.7	0.91	6.22
Serum albumin.				
Fraction A.	Sørensen (28).	4.8	0.67	3.79
" B.	"	4.8	0.61	4.22
" γ.	"	4.8	0.33	2.97
Carboxyhemoglobin.	Green and Cohn.†	6.6	0.55	2.71

* All of the results have been recalculated on the basis of the ionic strength per 1000 gm. of water.

† Personal communication.

as a result, β may vary in such a way as to alter the order of precipitation of a protein in a series arranged according to values of *Ks*. A diagram representing the precipitation of proteins at their respective isoelectric points would appear very different from a diagram representing their precipitation at the same pH.

With the exception of egg albumin all of the proteins in Table X are blood proteins. Of them all fibrinogen has the highest value of *Ks*. The values of β of fibrinogen at pH 6.0 and 6.6 are 2.51 and 2.81, and β presumably becomes smaller at those acid reactions which are in the neighborhood of its isoelectric point. But,

whereas fibrinogen, egg albumin, and serum albumin have acid isoelectric points, the isoelectric point of pseudoglobulin is at pH 5.44 and of hemoglobin at 6.78. The minimal solubility of egg albumin is near pH 4.5 and of hemoglobin near pH 6.6. Acidification from 6.6 to 5.4 increases the solubility of hemoglobin, but decreases that of the other proteins considered. Further acidification, let us say to pH 5.0, increases the solubility of serum globulin as well as hemoglobin, whereas the solubility of albumin and presumably of fibrinogen diminishes. At pH 6.6 hemoglobin precipitates over approximately the same range as serum globulin. At 5.4 pseudoglobulin precipitates at lower, hemoglobin at higher concentrations. In how far the separations conventionally performed at a neutral reaction would be complicated at the isoelectric point of serum albumin must await further study. The

TABLE XI.

Values of β and Ks in the Equation Log $S = \beta - Ks\mu$, Defining Solubility of Fibrinogen in Concentrated Solutions of Different Salts.

Salt.	pH	*Ks*	β
NaCl	5.8	0.91	1.61
$(NH_4)_2SO_4$	6.0	1.33	2.51
	6.6	1.33	2.81
$KH_2PO_4 + K_2HPO_4$	6.6	1.95	3.09

behavior of fibrinogen, however, may be clearly postulated. It possesses the highest value of *Ks*, and values of β at pH 6.6 of the same order as the β of carboxyhemoglobin, minimal at this reaction. Preliminary investigations indicate, however, that β for fibrinogen becomes progressively lower, at least to beyond pH 5.0. The readily precipitable condition of this blood protein is thus manifest and its physiological significance demands consideration.

Solubility of Fibrinogen in Concentrated Solutions of Different Salts.

Ammonium sulfate is a precipitant for most proteins. The solubility of proteins in phosphate buffer mixtures of the same pH is very similar, but the lower solubility of the phosphates themselves has appeared to limit their usefulness. The value of *Ks* and of β

for carboxyhemoglobin in ammonium sulfate and in potassium phosphate is identical within the error of measurement. The same constants vary but slightly for fibrinogen at pH 6.6., as shown in Table XI.

In Table XI the values of Ks for fibrinogen in solutions of sodium chloride, ammonium sulfate, and potassium phosphate are contrasted, showing that the relative order of these salts is that ordinarily given for the Hofmeister series. Most proteins are not precipitated by the neutral chlorides, but the insolubility of fibrinogen in the presence of relatively concentrated solutions of these salts renders it ideal for the further investigation of interionic forces in solutions of neutral salts and proteins.

I wish to express my appreciation of the kind advice and assistance given me by Dr. Edwin J. Cohn throughout this investigation.

SUMMARY.

Fibrinogen is readily transformed even at room temperature and at neutral reactions to substances which are no longer precipitated by half saturation with sodium chloride. Conditions of acidity and of temperature have been determined, however, under which fibrinogen may be sufficiently purified of other proteins and its own denaturation products to yield a chemical individual of constant and reproducible solubility.

The solubility of fibrinogen in concentrated salt solutions is defined by the equation $\log S = \beta - Ks\mu$.

The values of β and of the salting out constant, Ks, have been determined at 25° for sodium chloride at pH 5.8, ammonium sulfate at pH 6.0 and 6.6, and for potassium phosphate at pH 6.6.

The results that have been obtained with ammonium sulfate at pH 6.0 and 6.6 are consistent with the postulate that Ks is a constant for a given protein and salt, independent, over wide ranges, of temperature and pH (5).

The amphoteric properties of the protein are reflected by the values of β. The variation of β from 2.81 at pH 6.6 to 2.51 at pH 6.0 suggests that fibrinogen has an acid isoelectric point.

A comparative study of the solubility of the different blood proteins in concentrated solutions of the same salt, $(NH_4)_2SO_4$, shows that, of all of them, fibrinogen has the highest value of Ks. This

study is thus a further contribution towards the description of the "salting out" of the blood proteins by neutral salts, in terms of physicochemical constants.

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CLINICAL CALORIMETRY.

XLV. PROLONGED MEAT DIETS WITH A STUDY OF KIDNEY FUNCTION AND KETOSIS.*

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CONTENTS.

INTRODUCTION.

Two normal men volunteered to live solely on meat for one year, which gave us an unusual opportunity of studying the effects of this diet. The term "meat," as used by us, included both the lean and the fat portions of animals. The subjects derived most of their calories from fat and the diet was quite different from what one, who uses the term "meat" as including chiefly lean muscle, would expect. Rubner (1) called attention to the fact that a man cannot live on meat alone because of the

* These studies were supported in part by a research grant from the Institute of American Meat Packers.

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physical limitation of the apparatus of mastication. He was evidently considering only lean meat as fat offers little difficulty.

It is well known that the Eskimos have lived on an almost exclusive meat diet for generations. Certain explorers in the North also have subsisted for long periods on meat. Dr. Vilhjalmur Stefansson in particular has demonstrated that it is feasible for travelers in the arctic region to "live off the country," which means living on meat alone. The experiences of Stefansson and his companions have been given in his book "The Friendly Arctic" (2). He spent over 11 years in arctic exploration, during 9 years of which he lived almost exclusively on meat. Stimulated by this experience, Stefansson and Andersen, the latter a member of one of the expeditions, voluntarily agreed to eat nothing but meat for 1 year while they continued their usual activities in the temperate climate of New York.

LITERATURE.

The literature apparently contains no report concerning metabolic observations on subjects who lived exclusively on meat for long periods.

August and Marie Krogh, in 1908 (3), studied the dietary of the Greenland Eskimo. On the basis of the total annual food consumption of a section of Greenland, as collected by Rink in 1855, they estimated that the daily diet contained approximately 280 gm. of protein, 135 gm. of fat, and 54 gm. of carbohydrate. Thomas (4), finding no elevation of blood pressure and rarely any evidence of renal disease in the examination of 142 Eskimos, concluded that a carneous diet under strenuous living conditions does not produce renal or vascular disease. Heinbecker, in 1927 (5), found that the Baffin Island Eskimo, on a similar diet to that of the Greenland Eskimo, showed a normal carbohydrate tolerance, normal amounts of non-protein nitrogen in the blood, and no acetonuria. Fasting induced acetonuria in 3 days. In a recent discussion of high and low protein diets, Du Bois (6) referred to Catlin's statement that buffalo meat was the "staff of life" of the Central Plains' Indians. He cited also Head's account of subsisting on the native diet of beef and water while crossing the South American Pampas. All of these races are noted for their endurance of exertion and hardships.

Thomas (4) found no rickets or scurvy among the Greenland Eskimos, but a large incidence of these diseases among the Labrador Eskimos who live mostly on preserved food including dried potatoes, flour, canned foods, and cereals. Stefansson (7) reported three patients with scurvy on his last expedition, one of whom was our subject, Andersen. These cases were caused by eating canned foods with only a small amount of cooked meat, and were cured by eating raw meat. Lieb, in 1922 (8), carefully examined

Stefansson and stated that he found no evidence of injury from the prolonged and exclusive use of meat. He mentions the experience of two other men who lived mainly on meat for shorter periods.

Plan of Investigation.

The general scope of the investigation was outlined in 1926 and 1927 by an advisory committee of scientists of which Dr. Raymond Pearl of Johns Hopkins University, Baltimore, was chairman. The main portion of the work was carried on while the two subjects lived and ate in the metabolism ward of the Russell Sage Institute of Pathology in Bellevue Hospital, New York. The study was a cooperative one and several papers on various phases of the work have already been published. Short reports on the clinical features and general laboratory findings have been made by Lieb (9) and by McClellan (10). The excretion of acetone bodies has been discussed in papers on ketosis by McClellan, Spencer, Falk, and Du Bois (11) and by McClellan and Toscani (12). The chemical studies on the constituents of the blood have been presented by Lieb and Tolstoi (13) and by Tolstoi (14, 15). References to the above papers will be made later in this report.

A series of three papers, of which this is the first, will present the observations made in the ward, calorimeter, and laboratories of the Russell Sage Institute of Pathology.

PROTOCOLS.

V. Stefansson was an arctic explorer, aged 48, Canadian, and single. He had had measles at 10 years and mumps in early adult life and had survived typhoid fever, complicated by pneumonia as well as two attacks of pleurisy, which occurred in 1918, while on the arctic coast of Canada. For the past 10 years he had required occasional catharsis for moderate constipation, but except for infrequent colds, had not been ill during this period.

Physically, he was a well developed man, although his muscles were soft and somewhat flabby. His teeth contained many fillings and a mild gingivitis was present. The heart, lungs, and abdomen were normal. No changes in the blood vessels were detected.

Preliminary observations while on a mixed diet were started in the metabolism ward, February 13, 1928. Stefansson took his meals in the ward but slept at home. On February 26, 1928, he was admitted to the ward and on February 28, started on the meat diet. At our request he began eating lean meat only, although he had previously noted, in the North, that very lean meat sometimes produced digestive disturbances. On the 3rd day nausea

and diarrhea developed. When fat meat was added to the diet, a full recovery was made in 2 days. This disturbance was followed by a period of persistent constipation lasting 10 days. The subject had a craving for calf brain of which he ate freely. On March 12, poor appetite, nausea, and abdominal discomfort were present and a second but milder attack of diarrhea occurred which responded quickly to a proper proportionment of lean and fat meat. Because of the intestinal disturbance, the food intake for this period was below the average. Between March 21 and April 9, duplicate food samples were not saved for analysis. During this period the man's appetite was excellent. From April 10 to 19, duplicate diets were saved and complete analyses made. The subject left the hospital April 20 and continued the meat diet at home. One gastrointestinal disturbance occurred during the summer, from eating infected meat when abdominal pain, nausea, and vomiting were suffered for 36 hours. A table companion suffered from a similar but more severe attack. While on the meat diet, the explorer had two attacks of pharyngitis, one in the summer and one in December, 1928, while lecturing in the Middle West. Each attack subsided quickly without complications.

For the concluding observations Stefansson lived at home, where his diets were carefully checked by one of our own dietitians, but he came to the metabolism ward for calorimeter observations and blood studies. This terminal period of 8 days ended March 8, 1929, when he had completed 375 days on an exclusive meat diet. Then followed two periods of 1 week each —the first on a high fat diet, and the second on a general mixed diet. The investigations were concluded March 22, 1929.

K. Andersen was a fruit grower, born in Denmark, aged 38, and single, who had spent 5 years in arctic exploration and since 1920 worked an orange grove in Florida. His appendix had been removed 15 years previous.

The younger man was a thin, well developed adult with strikingly tanned skin and almost bald. The natural teeth which he still possessed—nine lower and seven upper—showed much repair, and he wore a denture replacing the four upper incisors. No gingivitis was evident. A soft, puffing, systolic sound was heard over the heart at mid-inspiration only, which was classed as a cardiorespiratory murmur.

Andersen entered the ward January 6, 1928, and started with a preliminary period on a mixed diet. The meat diet began January 24. No intestinal disturbances occurred. Intensive study on the effect of this régime continued for 90 days—until April 22, 1928. Our subject then left the hospital and, until the terminal observations, lived in a suburb, where he took plenty of exercise, while spending part of his time writing. In April and in August he had mild attacks of pharyngitis which lasted 2 to 3 days each. He reentered the ward on January 4, 1929, for our concluding studies. Meat was continued for 3 weeks, until January 24, making a total of 367 days on this diet. The next 3 weeks included three periods during which he received a variety of diets, all high in fat content. February 13 and 14, he had a mild pharyngitis. On the morning of February 15, he took 100 gm. of glucose for a glucose tolerance test. That evening he

had pain in the right chest, a severe chill, and rapidly rising temperature. The next day the sputum was rusty in color, the temperature was 40.0° (104°F.), and signs of consolidation were present over the right lower lobe. A diagnosis of lobar pneumonia was made. The infecting organism was the pneumococcus, Type II. Commencing 18 hours after the onset, Dr. Felton's concentrated polyvalent pneumococcus serum (Types I and II) was given in large amounts, and the temperature returned to normal at the end of 72 hours. Pain and general discomfort persisted for 5 days. General toxemia was slight, and convalescence was uneventful. While this infection lasted, the diet, mainly fluids at first, was rich in carbohydrate. Urine collections were continued throughout these periods but fecal specimens were not saved because medicated enemata were used. Following his convalescence, a period of 10 days on mixed diet, ending March 20, 1929, terminated the observations on Andersen. He left the hospital in good physical condition.

E. F. D. B. was a physician, American, married, aged 45, and in excellent health. During observation, he lived at home and came to the ward for his meals and the calorimeter studies. This subject went through a preliminary period on a general diet lasting 4 days, then for 10 days he ate nothing but meat. His appetite was poor and he required a number of days in which to adjust himself to the strange diet.

Nature of Diet.

The meat used included beef, lamb, veal, pork, and chicken. The parts used were muscle, liver, kidney, brain, bone marrow, bacon, and fat. While on lecture trips V. S. occasionally ate a few eggs and a little butter when meat was not readily obtainable. The carbohydrate content of the diet was very small, consisting solely of the glycogen of the meat. The men, except during short periods of special observation, ate as much as they wanted and proportioned the lean meat to the fat as they desired. V. S., in 31 days of special diet in the ward in which he was free from digestive disturbances, took an average of 0.81 kilos of meat per day while K. A. for 110 days averaged 0.79 kilos per day. The protein content of the diet ranged from 100 to 140 gm., the fat, from 200 to 300 gm., and the carbohydrate from 7 to 12 gm. The caloric value varied from 2000 to 3100 calories per day. 15 to 25 per cent of the calories were derived from protein, 75 to 85 per cent from fat, and 1 to 2 per cent from carbohydrate. Details concerning the food eaten are presented in Table I. The data for the meat periods were obtained from analyses of portions of meat which duplicated as closely as possible the meat actually eaten. Details

TABLE I.

Food and Urine Analyses.

All data are given in average daily values.

Period No.	No. of days.	Food.				Urine.				Remarks on diet.
		Protein.	Fat.	Carbohydrate.	Calories from protein.	Volume.	Acidity.	Nitrogen.	Acetone bodies.	
Subject K. A.										
		gm.	*gm.*	*gm.*	*per cent*	*cc.*	*cc.*	*gm.*	*gm.*	
1	8	61.8	122.4	164.1	12.4	1194	155	8.3	0.0	Mixed.
2	10	138.2	231.5	8.4	20.6	1162	531	20.3	6.17*	Meat.
3	10	124.1	202.2	7.7	21.1	1013	470	19.1	5.09	"
4	10	126.3	252.7	8.7	17.8	1182	493	17.9	5.16	"
5	9	116.0	281.2	8.1	15.2	1220†	517	17.6	4.98	"
6	11	117.8	243.8	9.2	17.4	1247†	523	16.8	5.53	"
7	10	122.6	223.8	10.2	19.2	1097	546	17.8	5.47	"
8	10	114.4	215.8	8.7	18.6	1074	513	18.1	4.62	"
9	10	126.9	216.5	10.4	20.2	1120	581	19.3	5.10	"
10	10	110.4	162.3	8.9	22.7	1490	503	18.4	3.18	"
										Elapsed time, 8 mos.
11	10	133.5	208.2	10.2	21.7	1303		22.2	2.85	Meat.
12	10	135.2	198.1	9.6	22.7	1354		21.7	2.48	"
13	7	45.9	227.6	4.9	8.1	1124		10.9	7.18	High fat, low protein.
14	7	45.0	233.4	34.7	7.4	922		8.2	6.20	" " " " and carbohydrate.
15	7	103.1	206.7	8.1	17.8	1532		16.8	5.06	Meat.
16	12	50.4	111.4	160.3	10.9	1949		14.7	0.0‡	Mixed diet, pneumonia.
17	12	90.8	202.8	280.5	10.9	1700		11.9	0.0	" " convalescent.
18	10	78.1	144.6	207.9	12.7	1645		9.2	0.0	Mixed.
Average for Periods 2–12........	110	124.1	221.1	9.1	19.6					Meat periods.

Subject V. S.										
1	5	80.0	149.8	200.4	12.9	1260	278	10.8	0.0	Mixed.
2	4	181.0	152.2	11.8	33.6	1373§	601	28.3	0.63	Meat (lean).
3	9	101.6	188.6	7.4	18.9	1072†	573	18.1	6.22	Meat.
4	9	85.7	116.0	5.7	24.2	962	369	16.2	4.81	"
5	10					1092	395	16.6	3.00	" not analyzed.
6	10					1114	555	21.1	1.47	" " "
7	10	148.0	217.5	10.8	22.7	1219	642	22.1	1.39	"
										Elapsed time, 10 mos.
8	8	99.4	188.4	6.6	18.6	1364		18.2	0.45	Last period of meat.
9	7	74.4	236.0	34.7	11.6	854		11.0	0.42	High fat, low carbohydrate.
10	7	80.1	148.0	208.6	12.8	1653		9.0	0.0	Mixed.
Average for Periods 2–4, 7, 8......	40	117.1	175.8	8.2	22.3					Meat periods.
Subject E. F. D. B.										
1	4	No data for food intake.				1270		12.2	0.0	Mixed.
2	5					1454		20.2	1.43*	Meat.
3	5					1422		22.3	1.16	"
4	4					1100		13.4	0.0‖	Mixed.

* First 3 days omitted from average.

† Urine averages for periods of 10 days.

‡ Acetone present 1st day of period. Sugar present first 4 days.

§ Urine averages for period of 3 days.

‖ Acetone present 1st day of period.

of these analyses will be found in the following paper of this series. The remainder of the data was calculated from the tables of food analysis, published by Rose (16). In Period 4, V. S. was far below his average intake on account of the intestinal disturbances previously described.

In this experiment, it was found that boiled meat was preferred to fried. Broiled steaks and chops were used,—V. S. choosing lamb frequently while K. A. ate beef almost exclusively. The meat was usually cooked lightly and the bone marrow eaten raw. Raw frozen meat was requested as a variation but no method of freezing it was available. The men generally took three but sometimes four meals daily. A sample menu for the day, given in raw weights follows.

Breakfast: lean beef, 190 gm.; fat, 100 gm.
Dinner: liver, 200 gm.; fat, 75 gm.
Supper: lean beef, 200 gm.; marrow, 70 gm.

The meat was usually cut from the bone and trimmed before weighing and cooking. In Period 7, V. S. ate "from the bone" which was the method of choice of both men, but this made the sampling of meat for analysis more difficult. The meat used during the first 3 months was selected from freshly killed animals; for the remainder of the time refrigerated meat from local markets was eaten.

The meat diet contained about 25 per cent of the amount of calcium found in the average mixed diet, while phosphorus and sulfur were present in larger quantities than usual. Table salt was allowed as desired but the men consumed only 1 to 5 gm. daily including that used in the cooking. As meat is one of the foods contributing to the acid portion of a ration, the diet was acid in the extreme but no calculation of the acid-base balance was made.

The daily intake of liquids—coffee, black tea, meat broths, and water, varied from 1 to 2 liters. When K. A. had pneumonia, however, he took 2.5 to 3.5 liters per day.

Did these men adhere strictly to the diet? We can answer in the affirmative with confidence for three reasons. First, the subjects were under close observation during a large part of the intensive studies. Second, in every individual specimen of urine which was tested during the intervals when they were living at

home, acetone bodies were present in amounts so constant that fluctuations in the carbohydrate intake were practically ruled out. Finally, the high character of the subjects is a guarantee that they faithfully followed the prescribed diet.

Effect of Diet on Clinical Condition.

General.—Both men were in good physical condition at the end of the observation. There were no subjective or objective evidences of any loss of physical or mental vigor. The teeth showed no deterioration and gingivitis had disappeared. There was, however, an increase in the deposit of tartar on teeth of V. S. Bowel elimination was undisturbed—V. S. required no extra catharsis and K. A. was regular throughout. The stools were

TABLE II.

Weights Given in Kilos.

Subject.	Beginning of observation.	Start of meat diet.	After 1 wk.	After 1 mo.	After 2 mos.	After 1 yr.	Pneumonia.		End of observations.
							Onset.	After recovery.	
V. S.	73.0	72.2	70.2	68.0	69.0	69.4			69.7
K. A.	60.0	59.4	58.3	58.5	60.5	58.0	55.6	56.1	56.6
E. F. D. B.	76.5	76.0	73.2						

smaller than usual, well formed, and had an inoffensive, slightly pungent odor. No flatus was noted.

Nutrition.—All three subjects lost weight during the 1st week of the meat diet—V. S., 2.0 kilos; K. A., 1.1 kilos; and E. F. D. B., 2.8 kilos. V. S. lost 2.2 kilos more in the next 3 weeks because of reduced intake at the time of his intestinal disturbance. After the 1st month both V. S. and K. A. regained part of the weight which they had lost and continued through the year with little variation. K. A. lost 2.4 kilos during the 3 weeks following the meat diet as the diets high in fat caused a loss of appetite resulting in diminished intake. Table II shows the weights of the subjects at various stages of the observation. The loss of weight in the 1st week is explained by a shift in the water content of the body which was adjusting itself to the different type of food eaten. The other losses of weight were definitely associated with dimin-

ished food intake. There was no evidence, as judged by weight, that the meat diet was detrimental to nutrition.

Blood Pressure.—The meat diet did not cause any elevation in the blood pressure of these two men despite the popular view that meat is a definite factor in producing such a result. V. S. maintained a systolic pressure of 105 mm. and a diastolic pressure of 70 mm. throughout the entire period. K. A. registered 140 mm. systolic and 80 mm. diastolic pressure at the beginning and 120 mm. systolic and 80 mm. diastolic pressure at the end of the year. The individual variations in pressure during the year were not significant.

Mental Attitude Toward Diet.—The two explorers who had lived on similar diets before, exhibited no mental reserve while eating meat exclusively. When the proportions of foodstuffs were correct, they ate with relish and no disturbances occurred. The third subject, a laboratory worker, had some difficulty in accustoming himself to such a diet and it required nearly a week to make the adjustment. He appeared to have some doubt regarding the outcome. This may have been due both to a lack of experience with such a diet and to the fact that his responsibilities were more pressing than those of other subjects.

Activity.—The men led somewhat sedentary lives. V. S. was writing, lecturing, and taking short walks daily. K. A. did some writing and walked 3 to 5 miles daily. Their response to muscular exertion was studied three times during the early part of the observations. The test consisted of running about $2\frac{1}{4}$ miles in approximately 20 minutes. Records of blood pressure and pulse rate while lying down were made before and after the run and were continued until both blood pressure and pulse rate returned to the resting levels. The blood pressure returned to its previous level in 10 to 15 minutes but in all three subjects the pulse rate required approximately 30 minutes to reach its resting level. No significant variation in response to this test was detected after 2 months of the meat diet.

According to their own reports the men carried on their usual activities without any increase of fatigue while taking meat. No unusual discomfort from the heat during the summer months was noted.

Intestinal Function.—The men were not troubled by consti-

pation more than when eating mixed diets. The diet was small in bulk and well absorbed (see the following paper of this series). In one instance, when the protein was relatively high, diarrhea developed. Fat aided in the regulation of the bowels. High fat diets have been used with success at the Mayo Clinic in the treatment of constipation (Smith (17, 18)). A detailed analysis of the second period of V. S. has been made to determine, if possible, whether any dietary factor was the cause of his intestinal upset (see protocols). Table III presents the food data for each day of this period. During the first 2 days his diet approximated that of the Eskimos, as reported by Krogh and Krogh (3), except that

TABLE III.

Daily Food Intake of V. S. during Period 2.

Date.	Intake.				Per cent of calories from:			Nitrogen.		Remarks.
	Protein.	Fat.	Carbohydrate.	Calories.	Protein.	Fat.	Carbohydrate.	Food.	Urine.	
1928	*gm.*	*gm.*	*gm.*					*gm.*	*gm.*	
Feb. 28	270	141	17	2489	44.6	52.6	2.8	43.2	24.5	Started meat diet.
" 29	257	137	16	2395	44.0	53.2	2.8	41.2	35.2	Weakness, nausea, and diarrhea.
Mar. 1	104	157	7	1916	22.2	76.3	1.5	16.6	25.2	
" 2	93	174	7	2028	18.8	79.8	1.4	14.9	21.3	Nausea and diarrhea absent.

he took only one-third as much carbohydrate. The protein accounted for 45 per cent of his food calories. The intestinal disturbance began on the 3rd day of this diet. During the next 2 days he took much less protein and more fat so that he received about 20 per cent of his calories from protein and 80 per cent from fat. In these two days his intestinal condition became normal without medication. Thereafter the protein calories did not exceed 25 per cent of the total for more than 1 day at a time. The high percentage of calories from protein may have been a factor in the production of the diarrhea.

Vitamin Deficiency.—No clinical evidence of vitamin deficiency

was noted. The mild gingivitis which V. S. had at the beginning cleared up entirely, after the meat diet was taken.

Effect of Diet on Urinary Constituents.

The daily determinations on the urine included volume, acidity, specific gravity, total nitrogen, total acetone bodies, albumin, and glucose. The Kjeldahl method was used for the total nitrogen and the method of Van Slyke (19) for computing the acetone bodies. The acidity was measured by the amount of 0.1 N alkali required to neutralize the 24 hour amount. A tabulation of these data by periods, computed as averages per day, is presented in Table I.

While meat was eaten, the average output of urine varied from 900 to 1500 cc. There were indications that some of the changes in urine volume were associated with changes in the amount of carbohydrate in the tissues. With two of the subjects (V. S. and E. F. D. B.) the urine volume was greater in Period 2, when carbohydrate was first omitted, than it was in the preceding period. In all three subjects it was at its lowest level in the period when carbohydrate was first added. A carbohydrate diet seems to favor retention of water in the body. The increased output of K. A. in Periods 16 and 17 paralleled the greater intake of fluids during his pneumonia.

The specific gravity ranged from 1012 to 1032 with the majority of the readings above 1020. The acidity of the urine showed a 2- to 3-fold increase during the meat diet as compared with the acidity of the preliminary period on mixed diet. This finding reflects the distinctly acid nature of the diet.

The average daily nitrogen excretion per period varied from 16.2 to 28.3 gm. V. S. showed the maximum excretion for a single day of 35.2 gm., on February 29, 1928, when eating purely lean meat. The acetone bodies in daily averages per period remained between 0.4 and 7.2 gm. K. A. showed the maximum excretion for one day of 12.3 gm., on February 13, 1928. No albumin, casts, or blood cells were found at any time.

Glucose appeared in the urine of K. A., February 15, 1929, and was present for 4 days. The daily amounts were 9.9, 17.0, 10.7, and 5.0 gm. This finding coincided with the giving of 100 gm. of glucose for a tolerance test and with the first 3 days of his pneumonia.

Effect of Diet on Kidneys.

Newburgh and his associates (20, 21) have reported that meat in large amounts is irritating to the kidneys of both animals and human beings. The clinical tests carried out on two of our subjects (V. S. and K. A.) revealed no evidence of irritation to the kidneys nor of damage to the kidney function. In the absence of microscopical studies of the kidneys themselves, one cannot say that no changes took place. Attention has been called to the fact that the diet was not particularly high in protein, being only 30 to 50 per cent above the average protein intake of the men when they partook of mixed diets.

The observations on which the above statements are based include the following.

TABLE IV.

Van Slyke Urea Clearance Tests.

Subject.	Date.	Blood urea N.	Excretion as per cent of normal.	Remarks.
	1929	*mg. per 100 cc.*	*per cent*	
K. A.	Feb. 13	12.6	105	End of meat diet.
	Mar. 15	18.5	42	After 4 wks. on mixed diet and after pneumonia.
V. S.	" 7	15.5	102	End of meat diet.
	" 19	11.3	76	After 2 wks. on mixed diet.

1. Examination of Urine.—No albumin, blood cells, or casts were observed at any time. The specific gravity showed good concentration throughout the year.

2. Phenolsulfonephthalein Excretion.—K. A. excreted 55 per cent of the dye in 2 hours and 10 minutes at the beginning and 62 per cent at the end. V. S. excreted 50 per cent at the beginning, but the concluding observation was not completed.

3. Urea Clearance Tests.—This test, which determines the amount of blood cleared of urea per minute, based on the urea excreted by the kidneys in the same time, has been described by Möller, McIntosh, and Van Slyke (22). Two tests were made on V. S. and two on K. A. by Dr. R. R. Hannon of The Rockefeller Institute Hospital to whom we are indebted for the following

data (Table IV). The first test on each subject, made at the end of 1 year on the meat diet, showed an excretion of urea which was above the average for normal men on mixed diets. The second test, made after a period on mixed diets was below the normal range for K. A. and at low normal for V. S.—the second test on K. A. was carried out after he had had pneumonia. A decrease in function following a period of hyperfunction has been noted in other conditions.

4. Blood Constituents.—Tolstoi (14) found no increase in the non-protein content of the blood of our subjects. He noted a slight increase of the uric acid nitrogen during the first 3 months, which was not present in the remainder of the observations. There was no change in the other non-protein nitrogenous constituents. Therefore, it may be stated that no retention of nitrogen occurred as a result of the meat diet. Baumgartner and Hubbard (23) found slight elevations of the urea nitrogen in the blood of two men who received diets containing 100 to 120 gm. of protein. This was noted early in their studies which lasted from 4 to 6 months. As the level was normal during the later months, they concluded that the diet did not damage the kidneys.

5. Size.—Roentgenographic studies of the kidneys by Dr. H. M. Imboden, at the beginning and the end of the meat diet, gave no evidence of kidney hypertrophy.

Effect of Diet on Fat Metabolism.

The diet used in these investigations contained a large proportion of fat calories and striking effects were noted in the utilization of this foodstuff. On the 1st day of the meat diet, acetone bodies were excreted in the urine showing that the amount of carbohydrate oxidized had already become insufficient to assure the complete combustion of the fat. Increasing amounts of acetone bodies were eliminated daily until the 4th day after which they remained nearly uniform in the case of K. A. but decreased steadily with V. S., throughout succeeding periods. Chart I shows the amounts of acetone bodies excreted in given periods. K. A. had a sustained level all through the first 3 months but at the end of the year, in Periods 11 and 12, he excreted about one-half as much as during the earlier intervals. In Period 13 the fat was proportionately higher than previously, due to the restriction of

protein. Here occurred the highest excretion of acetone bodies, an average of 7.2 gm. per day for a week. The acetonuria persisted while the subjects were receiving 5 per cent or less of their food calories from carbohydrate, but promptly disappeared when diets containing 30 per cent of the calories in carbohydrate were started—lasting only 4 hours in V. S., 12 hours in E. F. D. B., and 36 hours in K. A. The longer time required in the case of K. A. may have been due to the pneumonia which developed at that time.

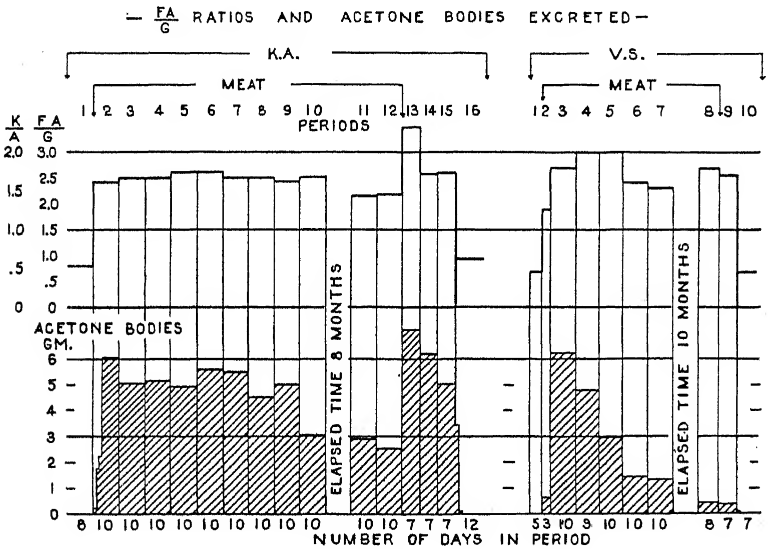

CHART I. A comparison of the fatty acid-glucose ratios and the acetone bodies excreted.

The blood showed a visible lipemia when the fat intake was at the higher levels and the cholesterol varied from 200 to 800 mg. per 100 cc. of blood reaching the higher values when the lipemia was noted (Tolstoi (14)). Total blood fats and blood acetone bodies were not determined.

Computation of the fatty acid-glucose ratios was made, using the Woodyatt formula (24). The estimated food metabolized was the basis of this analysis. Detailed data of the early periods

of the meat diet and the method of estimating the foodstuffs metabolized have been reported by McClellan and others (11) with a discussion of the threshold of ketosis in various conditions. The completed computation is presented in Chart I. It was found that acetone bodies were present in the urine when the FA:G ratios were above 1.5, but that the amount of acetone bodies excreted revealed no definite relation to the FA:G ratio. In K. A., the quantity showed a tendency to decrease even though the ratio remained relatively uniform and in V. S., the excretion decreased rapidly even while he was utilizing about the same amount of fat. In E. F. D. B., the excretion of acetone bodies remained low throughout the 10 days. These individual variations are difficult to explain. The subject, K. A., who had the high sustained ketosis, was the smallest of the three and had less subcutaneous fat than either of the others.

A comparison of the data supplied in Periods 8 and 9 of V. S. shows that for the protection of the body against ketosis, glucose derived from ingested protein is equivalent to glucose derived from carbohydrate food. In Period 8, when taking the meat diet, he was metabolizing foodstuffs with a FA:G ratio of 2.68 and excreting 0.45 gm. of acetone bodies per day. In Period 9, the diet was changed and contained less protein and 35 gm. of carbohydrate, which approximately equaled the available glucose contained in the omitted protein. The ratio for Period 9 was 2.54, and 0.42 gm. of acetone bodies were excreted daily.

No symptoms were noted, which could be attributed to the mild ketosis. There was no depression of mental faculties and no significant change in the carbon dioxide-combining power of the blood. We had no way of telling whether or not any changes had occurred in the walls of the blood vessels, but as far as clinical observations and special tests revealed, no injuries resulted from the prolonged mild ketosis.

SUMMARY AND CONCLUSIONS.

1. Two men lived on an exclusive meat diet for 1 year and a third man for 10 days. The relative amounts of lean and fat meat ingested were left to the instinctive choice of the individuals.

2. The protein content varied from 100 to 140 gm., the fat from

200 to 300 gm., the carbohydrate, derived entirely from the meat, from 7 to 12 gm., and the fuel value from 2000 to 3100 calories.

3. At the end of the year, the subjects were mentally alert, physically active, and showed no specific physical changes in any system of the body.

4. During the 1st week, all three men lost weight, due to a shift in the water content of the body while adjusting itself to the low carbohydrate diet. Thereafter, their weights remained practically constant.

5. In the prolonged test, the blood pressure of one man remained constant; the systolic pressure of the other decreased 20 mm. and the diastolic pressure remained uniform.

6. The control of the bowels was not disturbed while the subjects were on prescribed meat diet. In one instance, when the proportion of protein calories in the diet exceeded 40 per cent, a diarrhea developed.

7. Vitamin deficiencies did not appear.

8. The total acidity of the urine during the meat diet was increased to 2 or 3 times that of the acidity on mixed diets and acetonuria was present throughout the periods of exclusive meat.

9. Urine examinations, determinations of the nitrogenous constituents of the blood, and kidney function tests revealed no evidence of kidney damage.

10. While on the meat diet, the men metabolized foodstuffs with FA:G ratios between 1.9 and 3.0 and excreted from 0.4 to 7.2 gm. of acetone bodies per day.

11. In these trained subjects, the clinical observations and laboratory studies gave no evidence that any ill effects had occurred from the prolonged use of the exclusive meat diet.

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CLINICAL CALORIMETRY.

XLVI. PROLONGED MEAT DIETS WITH A STUDY OF THE METABOLISM OF NITROGEN, CALCIUM, AND PHOSPHORUS.

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CONTENTS.

Analysis of Meat.

Two arctic explorers lived for 1 year on a diet consisting exclusively of meat, as described in a previous communication (1). In order to analyze their diet, we duplicated, as nearly as possible, the food taken by them while they were in the metabolism ward. The meat consumed during the earlier periods of observation was from animals killed the preceding day, but after the 3rd month, ordinary refrigerated meat was used. The samples were put into closed but not air-tight containers, and kept in an ice box for periods of 3 to 10 days. Analyses were then made of aliquot portions of the collected meat samples for each period. Determinations of nitrogen and fat were made on twenty individual samples of muscle and six of tongue.

The lean meat was weighed, ground in a meat grinder, and an aliquot portion dried on a steam bath. The dried material was weighed, passed through a grinder to break up the lumps, and stored in Mason jars. It was further pulverized in a mortar before samples were taken for analysis. For nitrogen determinations, duplicate samples of 0.5 gm. each of the dried material were used. These were digested with a mixture consisting of 50 cc. of 5 per cent $CuSO_4$ solution, 300 cc. of 85 per cent phosphoric acid, and 100 cc. of concentrated sulfuric acid. The Kjeldahl method was followed. Pumice was added to reduce bumping. Fat determinations were made on 5.0 gm. portions of the dried meat by extracting them overnight in a Soxhlet apparatus with ethyl ether and purifying the extract with petroleum ether.

TABLE I.

Analyses of Meat: Nitrogen and Fat.

Gm. per 100 gm. wet weight.

Nature of meat.	No. of samples.	Nitrogen.			Fat.		
		Maximum.	Minimum.	Average.	Maximum.	Minimum.	Average.
Beef muscle (well trimmed).	27	3.77	2.78	3.24	7.80	2.50	5.02
" " (not ").	9	3.60	2.84	3.18	20.80	7.70	11.78
" tongue	17	3.65	2.70	3.15	34.60	14.70	24.24
" liver	14	3.34	2.61	2.97	8.55	3.58	5.85
" kidney	2	2.47	2.23	2.35	2.80	2.40	2.60
" brain	1			1.68			9.30
" fat	18	0.34	0.17	0.22	92.70	86.00	90.20
" bone marrow	8	0.25	0.15	0.20	90.30	87.80	89.10
Veal	2	3.00	2.76	2.88	17.21	13.40	15.31
Lamb	4	2.56	2.23	2.42	33.00	22.90	26.85
Bacon	5	1.40	1.08	1.23	63.00	57.50	59.50

Samples of fat and marrow were passed through a meat grinder, three or four times, and aliquot portions were preserved in the ice box. The true fat content of these samples was determined as follows: Approximately 10 gm. of material were weighed in a Petri dish and dried on a steam bath to constant weight, thus the water content of the fat was obtained. Warm gasoline was added to dissolve the fat, and the non-soluble matter was separated by use of a Gooch crucible. The residue in the crucible was

washed several times with warm gasoline and finally with ether. The crucible was dried to constant weight and the non-soluble matter weighed. After the weight of the water and of the non-soluble ingredients was deducted from the original weight of the sample, the remainder was taken as the true fat content. The material which was insoluble in ether was collected from a number of samples and analyzed for its nitrogen content which was found to be close to 10 per cent. This figure was used in calculating the nitrogen content of the fat after determining the residue which was insoluble in ether. The same procedure was adopted with the marrow.

TABLE II.

Analyses of Meat: Calcium and Phosphorus.

Gm. per 100 gm. wet weight.

Nature of meat.	No. of samples.	Calcium.			Phosphorus.		
		Maximum.	Minimum.	Average.	Maximum.	Minimum.	Average.
Beef muscle	17	0.0180	0.0084	0.0118	0.260	0.194	0.223
" tongue	9	0.0091	0.0063	0.0075	0.214	0.181	0.201
" liver	13	0.0120	0.0058	0.0079	0.480	0.310	0.416
" kidney	1			0.0074			0.252
" brain	1			0.0076			0.380
" fat	5	0.0190	0.0100	0.0138	0.065	0.050	0.056
Veal	2	0.0146	0.0134	0.0140	0.216	0.191	0.204
Lamb	4	0.0330	0.0109	0.0210	0.190	0.170	0.181
Bacon	5	0.0187	0.0096	0.0132	0.120	0.090	0.108

Phosphorus was determined in samples of 1.0 gm. each by the volumetric molybdate method after digestion with nitric and sulfuric acids (2). Calcium was determined by McCrudden's method (3) on 10 gm. of dried material which had been ashed.

Table I contains the maximum, minimum, and average values for nitrogen and fat. The data for calcium and phosphorus are given in Table II. A large variation was found in the fat content of different samples of tongue, according to the portion of the tongue used, the base having the highest fat content. The muscle samples in the first group were very lean as all free fat was carefully trimmed off in the ward kitchen; those in the second group had a higher fat content because they were not trimmed. There was

considerable variation in the amount of calcium in the different samples of the same kind of meat. This agrees with the findings of Heubner and Rona (4) and of Denis and Corley (5). The calcium content of meat as given by Sherman and Gettler (6) is 0.055 gm. of calcium per 100 gm. of protein. The average result of the seventeen determinations made by us on beef muscle was 0.059 gm. of calcium per 100 gm. of protein.

Absorption of Meat.

The absorption of food materials from the intestinal tract is an important feature in any study of nutrition. The unabsorbed portions play no part in the foodstuffs actually metabolized in the body. Prausnitz (7) found little variation in the amount of nitrogen in the feces with wide fluctuations in the amount of protein in the food. Bloor and his associates (8, 9) noted the presence of fat in the feces of animals on fat-free diets and emphasized the fact that such fat may come from sources other than the food ingested.

The feces were collected for periods of 3 to 10 days, preserved with acid-alcohol, and dried, and the nitrogen and fat determinations were made by the same methods as those used for food. No analyses for the amount of carbohydrate in the feces were made because the food contained only that carbohydrate which was present as glycogen in the meat. Identification of the nature of the nitrogen and fat found in the feces was not attempted. The significant data obtained are presented in Table III.

In the case of the subject K. A., the nitrogen loss in the feces showed a variation of 2.4 to 7.1 per cent with an average of 4.5 per cent during the periods when he was receiving meat alone. The subject V. S. lost from 6.5 to 10.4 per cent of his ingested nitrogen, an average of 7.5 per cent. A comparison with the control periods shows a better absorption of protein in K. A. while on the meat diet but no significant difference in V. S.

The loss of fat in K. A. varied between 1.7 and 4.8 per cent with an average of 3.0 per cent; the loss for V. S. ranged from 5.4 to 14.6 per cent with the average at 9.2 per cent. The absorption of fat in both subjects was essentially the same as during the control periods.

In the case of each subject, the percentage loss of both nitrogen

TABLE III.

*Percentage of Ingested Nitrogen and Fat Excreted in the Feces.**

Subject.	Period No.	No. of days.	Nitrogen. Food.	Nitrogen. Feces.	Nitrogen.	Fat. Food.	Fat. Feces.	Fat.	
			gm.	*gm.*	*per cent*	*gm.*	*gm.*	*per cent*	
K. A.	1	8	79.1	10.1	12.8	980	21.4	2.2	
	2	10	221.0	7.2	3.3	2315	44.6	1.9	
	3	10	198.5	10.7	5.4	2022	96.3	4.8	
	4	10	202.1	4.9	2.4	2527	43.6	1.7	
	5	10	185.8	13.1	7.1	2812	113.2	4.0	
	6	10	188.7	7.6	4.0	2438	59.5	2.4	
	7	10	196.1	9.5	4.8	2238	78.8	3.5	
	8	10	183.0	8.6	4.7	2158	67.1	3.1	
	9	10	203.1	7.7	3.8	2165	67.1	3.1	
	10	10	175.9	9.0	5.1	1623	41.7	2.6	
									Interval 8 mos.
	11	10	213.6	9.8	4.6	2082	60.0	2.9	
	12	10	216.3	8.7	4.0	1981	53.5	2.7	
	13	7	51.3	6.0	11.7	1593	103.0	6.5	
	14	7	50.4	5.7	11.3	1634	39.5	2.4	
	15	7	115.5	6.4	5.5	1447	33.0	2.3	
	16	12	96.8	17.6†	18.2	1337			
	17	12	174.5	14.3†	8.2	2434			
	18	10	125.0	13.3	10.7	1446	24.3	1.7	
Average per day, Periods 2–12.‡.....			19.9	0.9	4.5	221.5	6.6	3.0	
V. S.	1	5	64.0	5.4	8.4	750	82.5	11.0	
	2	4	115.9	7.7	6.6	609	65.0	10.7	
	3	9	146.2	9.8	6.7	1697	145.8	8.6	
	4	9	123.3	12.8	10.4	1044	152.2	14.6	
	5	10§							
	6	10§							
	7	10	236.8	17.2	7.3	2175	202.0	9.3	
									Interval 10 mos.
	8	8	127.3	8.3	6.5	1507	81.0	5.4	
	9	7	83.4	7.6	9.1	1652	21.8	1.3	
	10	7	89.8	12.6	14.0	1036	23.0	2.2	
Average per day, Periods 2–4, 7, and 8.‡...........			18.7	1.4	7.5	175.8	16.2	9.2	

* The explanatory remarks in Table IV, apply to periods as given in this table.

† Estimated as 10 per cent of the urinary nitrogen as found when on mixed diet in Periods 1 and 18.

‡ Exclusive meat diet.

§ Data for these periods are incomplete.

and fat remained nearly the same throughout the different periods. There was a wide difference, however, between the results in the

TABLE IV.

Nitrogen Balance.

Subject.	Period No.	No. of days.	Total in-take.	Output.			Balance for period.	Remarks.
				Urine	Feces.	Total.		
			gm.	*gm.*	*gm.*	*gm.*	*gm.*	
K. A.	1	8	79.1	65.3	10.1	75.4	+3.7	Mixed diet.
	2	10	221.0	202.6	7.2	209.8	+11.2	Meat.
	3	10	198.5	190.6	10.7	201.3	−2.8	"
	4	10	202.1	179.4	4.9	184.3	+17.8	"
	5	10	185.8	175.6	13.1	188.7	−2.9	"
	6	10	188.7	167.6	7.6	175.2	+13.5	"
	7	10	196.1	178.3	9.5	187.8	+8.3	"
	8	10	183.0	181.0	8.6	189.6	−6.6	"
	9	10	203.1	193.4	7.7	201.1	+2.0	"
	10	10	175.9	183.6	9.0	192.6	−16.7	"
	11	10	213.6	222.1	9.8	231.9	−18.3	"
	12	10	216.3	217.0	8.7	225.7	−9.4	"
	13	7	51.3	76.5	6.0	82.5	−31.2	Low protein, high fat.
	14	7	50.4	57.7	5.7	63.4	−13.0	" " " " 35 gm. carbohydrate.
	15	7	115.5	117.4	6.4	123.8	−8.3	Meat.
	16	12	96.8	176.2	17.6*	193.8	−97.0	Mixed diet, pneumonia.
	17	12	174.5	142.5	14.3*	156.8	+17.7	" " convalescent.
	18	10	125.0	92.3	13.3	105.6	+19.4	Mixed diet.
V. S.	1	5	64.0	54.2	5.4	59.6	+4.4	Mixed diet.
	2	4	115.9	106.2	7.7	113.9	+2.0	Meat.
	3	9	146.2	159.2	9.8	169.0	−22.8	"
	4	9	123.3	145.7	12.8	158.5	−35.2	"
	5	10						"
	6	10						"
	7	10	236.8	221.6	17.2	238.8	−2.0	"
	8	8	127.3	145.6	8.3	153.9	−26.6	"
	9	7	83.4	77.2	7.6	84.8	−1.4	Low protein, high fat, 35 gm. carbohydrate.
	10	7	89.8	63.1	12.6	75.7	+14.1	Mixed diet.

* Estimated as 10 per cent of the urinary nitrogen.

cases of the two individuals, although both were within normal limits. In each case, both nitrogen and fat were well absorbed.

Metabolism of Nitrogen, Calcium, and Phosphorus.

A meat diet, unless supplemented by some material, like bone, which is rich in calcium, is unsatisfactory from the mineral stand-

TABLE V.

*Calcium Balances.**

Subject.	Period No.	No. of days.	Total intake.	Output.			Balance for period.
				Urine.	Feces.	Total.	
			gm.	*gm.*	*gm.*	*gm.*	*gm.*
K. A.	1	8	2.950	1.784	1.360	3.144	−0.194
	2	10	0.611	3.131	1.088	4.219	−3.608
	3	10	0.555	4.107	1.645	5.752	−5.197
	4	10	1.540	3.978	0.950	4.928	−3.388
	5	10	1.346	3.060	2.420	5.480	−4.134
	6	10	0.975	3.252	1.108	4.360	−3.385
	7	10	1.011	3.012	1.192	4.204	−3.193
	8	10	1.034	3.360	1.147	4.507	−3.473
	9	10	0.713	3.190	1.088	4.278	−3.565
	10	10	0.932	2.842	0.855	3.697	−2.765
	11	10	1.167	2.550	0.977	3.527	−2.360
	12	10	1.178	2.300	0.631	2.931	−1.753
	13	7	0.789	1.932	1.000	2.932	−2.143
	14	7	2.420	3.440	1.290	4.730	−2.310
	15	7	0.637	1.770	0.590	2.360	−1.723
	16	12		3.800			
	17	12		3.800			
	18	10	5.826	3.080	3.540	6.620	−0.794
V. S.	1	5	2.172	0.339	1.158	1.497	+0.675
	2	4	0.496	0.392	1.148	1.540	−1.044
	3	9	0.716	1.228	1.588	2.816	−2.100
	4	9	0.625	1.069	2.015	3.084	−2.459
	5	10					
	6	10					
	7	10	1.166	1.470	7.910	9.380	−8.214
	8	8	0.644	0.784	1.320	2.104	−1.460
	9	7	2.451	1.360	1.083	2.443	+0.008
	10	7	3.431	0.789	2.194	2.983	+0.448

* The explanatory remarks as given in Table IV apply in this table.

point because of its low calcium content. Sherman (10) states that the normal calcium requirement of a man weighing 70 kilos is approximately 0.45 gm. per day. The calcium content of meat as

given by Sherman and Gettler (6) is 0.055 gm. of calcium per 100 gm. of protein. Therefore it would require about 800 gm. of protein per day to supply the needed calcium.

TABLE VI.

*Phosphorus Balance.**

Subject.	Period No.	No. of days.	Total intake.	Output.			Balance for period.
				Urine.	Feces.	Total.	
			gm.	*gm.*	*gm.*	*gm.*	*gm.*
K. A.	1	8	6.59	3.88	1.68	5.56	+1.03
	2	10	13.61	14.21	1.07	15.28	−1.67
	3	10	13.04	12.58	1.39	13.97	−0.93
	4	10	13.39	12.65	0.76	13.40	−0.02
	5	10	13.75	11.52	2.05	13.57	+0.18
	6	10	15.09	13.11	1.28	14.39	+0.70
	7	10	17.01	14.76	1.53	16.29	+0.72
	8	10	14.76	12.88	1.25	14.13	+0.63
	9	10	17.12	13.68	1.12	14.80	+2.32
	10	10	14.90	12.96	1.48	14.44	+0.46
	11	10	19.19	16.72	1.74	18.46	+0.73
	12	10	18.45	17.56	1.68	19.24	−0.79
	13	7	5.21	6.89	1.12	8.01	−2.80
	14	7	5.16	5.21	1.29	6.50	−1.34
	15	7	10.61	10.12	1.39	11.51	−0.90
	16	12		13.01			
	17	12		13.01			
	18	10	11.81	9.02	3.49	12.51	−0.70
V. S.	1	5	4.89	3.00	1.71	4.71	+0.18
	2	4	8.65	5.84	2.57	8.41	+0.24
	3	9	15.67	11.50	2.82	14.32	+1.36
	4	9	8.18	9.45	3.15	12.60	−4.42
	5	10					
	6	10					
	7	10	21.49	15.51	6.90	22.41	−0.92
	8	8	10.63	10.16	2.07	12.23	−1.60
	9	7	6.69	6.96	1.49	8.45	−1.76
	10	7	8.37	5.10	3.43	8.53	−0.16

* The explanatory remarks as given in Table IV apply in this table.

A meat diet contains an excess of the acid-forming elements, sulfur and phosphorus. The effect of an acid diet on the excretion of calcium has been reviewed by Stewart and Percival (11). More

recently Bauer, Albright, and Aub (12) have reported that an acid diet increases the calcium excretion and that the increase occurs principally in the urine.

The phosphorus metabolism with acid diets has not received as much consideration as the calcium metabolism. Fitz, Alsberg, and Henderson (13) gave rabbits a diet to which hydrochloric acid had been added and noticed first an increase and then a decrease in the output of phosphorus in the urine. Goto (14) noted an increase in the excretion of phosphorus in the urine of rabbits after the ingestion of acid-forming food.

The balances for the men which we studied are given in Tables IV to VI which contain the total intake and output for each period. The intake values for the meat periods were obtained by analysis as described in the first section of this paper; for the other periods, they were calculated from the tables prepared by Rose (15).

Nitrogen Balance.—While on the meat diet, K. A. was in nitrogen equilibrium. In Period 13, his appetite was poor, and he swung to the negative side, and in Period 16, when he had pneumonia, his balance was −8.0 gm. of nitrogen per day. V. S. had a definite negative balance in Periods 3, 4, and 8, while taking meat alone, which was partly accounted for by the digestive disturbances previously described (1) which resulted in a loss of appetite and diminished intake.

Calcium Balance.—In the preliminary period, the subject K. A. had a slightly negative calcium balance, with a daily intake of 0.369 gm. Sherman has stated that the average calcium requirement for a man is 0.45 gm. per day. It is interesting to note that the mean daily calcium excretion of this subject while receiving the meat diet was 0.44 gm.—the urinary excretion was roughly 3 times the amount excreted in the feces. In the preliminary period, V. S. had a positive balance with an intake of 0.434 gm. daily. His urinary excretion of calcium was considerably less than that of the other subject. Both men had definitely negative balances throughout the periods of meat ingestion. It was also noted that both the calcium excreted in the urine and the total output were greater than the amounts excreted when they were receiving mixed diets. This may have been due to the acid nature of the diet.

The supplementary intake of calcium which was not accurately controlled, had its sources in the drinking water, table salt, and small particles of bone taken with the bone marrow. The drinking water was found to contain approximately 0.01 gm. of calcium per liter. On the assumption that the subjects drank 2 liters of water per day, it would contribute 0.02 gm. of calcium daily or 0.2 gm., for a period of 10 days. Compared with a negative balance of 3.0 gm. of calcium, this would account for only 6 per cent of the loss. Analysis of the table salt used showed a content of 0.004 gm. of calcium in 1.0 gm. of salt. They took between 1.0 and 5.0 gm. of salt per day which would account for between 3 and 6 per cent of the loss. The calcium intake as reported in Table V does not include the calcium taken in water or table salt.

Both subjects received considerable quantities of bone marrow at various times and to obtain it the shafts of long bones were sawed in sections about 6 inches long and cracked open with a hammer. In this process, some splintering occurred and there was a possibility of removing a slight amount of the adjacent bone with the marrow. Marrow supplied in this way was divided in half; one part was eaten, and the other half was sent to the laboratory for analysis. Determinations, made on eleven samples of marrow, showed a variation in calcium content ranging from 0.043 to 0.092 gm. per 100 gm. of marrow. Eight of these determinations showed from 0.050 to 0.076 gm. of calcium per 100 gm. of marrow. As the samples for each period represented fairly accurately the marrow ingested in that period, the errors arising from this source are not significant. Several analyses of marrow that had been carefully removed with a spatula without cracking the bone or scraping the sides showed a mean calcium content of 0.005 gm. per 100 gm. of marrow. The increase in the calcium intake of the subject K. A. in the third and subsequent periods is due mainly to the calcium received in the bone marrow.

In Period 7, V. S. showed a 3-fold increase of calcium in the feces and an apparent increase in the calcium loss. The estimated intake of calcium and phosphorus during this period was undoubtedly much too low, as he ate the meat directly from the bone, sometimes chewing the soft ends of the ribs, while previously he was given meat which had been cut from the bones.

No evidence was obtained which indicated that the loss of cal-

cium from the body was in any way serious. Roentgenograms of the hands, when compared with those of men of approximately the same size who were receiving mixed diets, showed no rarefaction. The loss of calcium, observed during the intensive studies, may not have continued when they ate the meat from the bone.

Phosphorus Balance.—The phosphorus balance in the preliminary periods was positive for both subjects. With the higher intake of phosphorus during the meat periods, it varied from negative to positive. The increased phosphorus ingested was entirely eliminated in the urine. The average daily excretion in the feces decreased during the meat periods. The balance for the entire period of meat ingestion was +2.34 gm. for K. A. and −5.34 gm. for V. S. Throughout the period of observation the blood calcium and phosphorus remained normal (Tolstoi (16)).

SUMMARY AND CONCLUSIONS.

1. The results of the analyses of many samples of meat are presented. They agree in general with the findings in other available reports.

2. Observations were made to determine the efficiency of absorption of the foodstuffs from the intestinal tract. The loss of nitrogen in the feces of K. A. was 4.5 per cent of the intake and for V. S. was 7.5 per cent. The loss of fat for K. A. was 3.0 per cent and for V. S., 9.2 per cent.

3. The use of the meat diets by the two subjects studied did not reduce the efficiency of absorption from the intestinal tract.

4. The nitrogen, calcium, and phosphorus balances of the two men are presented.

5. One subject (K. A.) remained in nitrogen equilibrium while receiving meat with an average daily intake of 19.9 gm. and the other (V. S.) showed a slight negative balance with an intake of 18.7 gm. per day.

6. The daily intake of calcium was from 0.05 to 0.15 gm. The average daily excretion of calcium for one subject was 0.44 gm., and for the other 0.47 gm. Both men showed negative calcium balances when taking meat alone.

7. In spite of the high phosphorus intake, K. A. showed a positive phosphorus balance of only +2.34 gm. for the entire period of meat ingestion, while V. S. showed a balance of −5.34 gm. This

was due to the increased excretion of phosphorus in the urine since the phosphorus in the feces decreased when the meat diet was taken.

8. The influence of the acid nature of the diet on mineral balances has been discussed.

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THE DEAMINIZATION OF CYSTINE.

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In a previous paper (1) the writer reported some studies on the decomposition of cystine in the presence of alkalies. A part of this work was concerned with the rate of loss of ammonia when cystine is heated in alkaline solution and with the acceleration resulting from the presence of certain heavy metals, notably lead. More recently, Gortner and Sinclair (2) have reported further work on the same subject and while their experimental procedure differs somewhat from that of the writer (see below) a comparison of the two sets of data leads to some interesting questions as to the mechanism of this reaction.

In the paper by Andrews (1) the rate of deaminization in various concentrations of sodium hydroxide was studied. It was found that as the concentration of sodium hydroxide was increased from about 0.001 M up, the rate of deaminization increased to a maximum at about 0.2 M and then decreased as the concentration of alkali was carried higher. It was also found that the presence of amounts of lead equivalent to the sulfur present more than tripled the rate of ammonia formation whereas similar amounts of copper had a smaller effect and other heavy metals had practically none. In the paper by Gortner and Sinclair deaminization by means of other alkalies such as sodium carbonate, potassium hydroxide, and calcium, strontium and barium hydroxides was reported. Since some of these, particularly the alkaline earth hydroxides, gave surprizingly high rates of deaminization these authors question the specificity of lead in accelerating this reaction. Aside from the question of the effect of lead, these very pronounced results with the alkaline earths are so interesting as to invite attempts to show to what relative extents the alkalinity and the

concentration of specific ions operate on this reaction. Further studies are therefore reported below on these metals but with the procedure previously used by the writer.

This procedure differs from that of Gortner and Sinclair in that a more constant temperature was employed. The steam-jacketed container used was a Hopkins condenser with the 50 cc. of cystine solution placed in the inner tube and the steam carried through the outer from a gently boiling flask and condensed in a second condenser connected with the side arm of the Hopkins condenser. Here, the only variation in temperature is that occasioned by barometric fluctations whereas the practice of Gortner and Hoffman of boiling the solutions introduces the systematic error due to varying concentrations and correspondingly varying boiling points. The yield of ammonia, moreover, fluctuates considerably on direct boiling due to the effect of superheating, drafts of air on the flask, etc. It is felt, therefore, that the procedure employed by the writer produces more consistent results.

In Table I are reported the details of a series of deaminization experiments with cystine, in which, unless otherwise noted, the conditions mentioned above (0.25 gm. of *l*-cystine in 50 cc. of the solution, heated and swept for 5 hours) are used. All experiments were made with a repeatedly recrystallized sample of *l*-cystine with the theoretical content of both nitrogen and sulfur. No variations in the optical activity of the cystine were employed since the lack of any influence of this property on the rate of deaminization was reported previously by the writer and confirmed by Gortner and Sinclair. The figures reported for percentage deaminization are the average of at least two determinations with results varying by not more than $\pm$ 0.7 per cent.

The data reported in Table I were intended, in part, to elucidate the question of the specificity of lead salts in promoting the deaminization of cystine. Gortner and Sinclair point out that according to their tables "the decomposition of cystine in solutions of sodium carbonate, barium hydroxide, and strontium hydroxide is as great or greater than Andrews found when lead was present." However, if the percentage composition of the sodium carbonate solutions reported on by these authors is calculated in terms of normalities it is readily seen that the amount of decomposition effected by sodium carbonate is very close to that effected by the same normal-

ity of sodium hydroxide in the same length of time. This result is confirmed by the present writer (Table I, Experiments 1 to 4). However, the percentage decomposition obtained here is far less than that reported by the writer with lead present in sodium hydroxide solutions. Even in the sodium carbonate solutions with the lead precipitated as carbonate a striking increase in deaminization results (Table I, Experiments 5 to 7). On the other hand, the alkaline earth hydroxides, when employed alone do cause remarkably high deaminization figures in higher concentrations although 0.05 N barium hydroxide produces scarcely more ammonia than does 0.05 N sodium hydroxide (Table I, Experiment 44). Here again the addition of lead salts causes marked increases. In Experiments 45 to 52 various proportions of lead and barium salts are compared. From a comparison of Experiments 42, 47, and 48 it will be seen that to raise the percentage decomposition of cystine in a barium hydroxide solution a comparable amount, the mols of barium salt required are about 35 times that of lead. Many other similar comparisons are afforded by the data in Table I. The great effectiveness of calcium hydroxide in promoting deaminization of cystine has also been reported by Marriott (3) who investigated the deaminization effects of a number of alkaline solutions. Our results are in substantial agreement with those reported by him with one exception noted below.

A more extensive series of data on the use of potassium hydroxide is included to supplement the data previously published on sodium hydroxide. While it is true that potassium hydroxide causes somewhat less deaminization than does sodium hydroxide the differences are not great. A series of experiments (Experiments 16 to 37) also shows the relative effect of various added salts. It is evident that the effect of lead salts is incomparably greater than that of any of the others. It is particularly noteworthy that calcium and barium salts are so ineffective when used in this way. However, the addition of 0.005 mol of calcium tartrate to a 0.044 N $Ca(OH)_2$ solution (Experiment 55) produces a very substantial increase in the ammonia evolved.

The experiments suggested in some cases, the need of a threshold concentration of the alkaline earth metal. Furthermore, it is obvious that the 5 per cent $Ca(OH)_2$ used by Gortner and Sinclair in their Table III cannot be all in solution. Therefore, the effec-

TABLE I.

Percentage Deaminization of Cystine at 100°.

Experiment No.	Solution conditions.	Mols of salt added.	Average per cent deamination.
1	2.0 N NaOH		18.3
2	2.0 " Na_2CO_3		21.5
3	1.0 " "		18.5
4	0.50 " "		17.3
5	2.0 " " + 0.79 gm. $(CH_3COO)_2Pb \cdot 3H_2O$ (equivalent to S)	0.0021	27.1
6	1.0 N Na_2CO_3 + 0.79 gm. $(CH_3COO)_2Pb \cdot 3H_2O$	0.0021	23.5
7	0.50 " " + 0.79 " "	0.0021	23.8
8	3.65 " KOH		8.8
9	2.0 " "		16.4
10	1.0 " "		20.6
11	0.5 " "		23.6
12	0.25 " "		22.1
13	0.10 " "		19.3
14	0.05 " "		13.7
15	3.65 " " + 1.58 gm. $(CH_3COO)_2Pb \cdot 3H_2O$	0.0042	46.9
16	3.65 " " + 0.79 " "	0.0021	43.4
17	2.0 " " + 1.58 " "	0.0042	65.1
18	2.0 " " + 0.79 " "	0.0021	54.9
19	2.0 " " + 0.079 " "	0.00021	21.5
20	2.0 " " + 8.0 " $BaCl_2 \cdot 2H_2O$	0.033	40.4
21	2.0 " " + 5.0 " "	0.020	26.3
22	2.0 " " + 3.0 " "	0.012	21.0
23	2.0 " " + 1.222 " "	0.005	18.6
24	2.0 " " + 1.095 " $CaCl_2 \cdot 6H_2O$	0.005	17.9
25	2.0 " " + 0.52 " $CuSO_4 \cdot 5H_2O$	0.0021	33.4
26	1.0 " " + 1.58 " $(CH_3COO)_2Pb \cdot 3H_2O$	0.0042	76.5
27	1.0 " " + 0.79 " "	0.0021	64.8
28	1.0 " " + 0.079 " "	0.00021	25.7
29	1.0 " " + 2.92 " NaCl	0.050	21.4
30	1.0 " " + 1.22 " $BaCl_2 \cdot 2H_2O$	0.005	30.0
31	1.0 " " + 0.61 " "	0.0025	25.1
32	1.0 " " + 1.045 " $CaCl_2 \cdot 6H_2O$	0.0048	23.5
33	0.5 " " + 0.79 " $(CH_3COO)_2Pb \cdot 3H_2O$	0.0021	71.3
34	0.5 " " + 0.079 " "	0.00021	27.4
35	0.25 " " + 0.079 " "	0.00021	26.6
36	0.10 " " + 0.079 " "	0.00021	25.0
37	0.05 " " + 0.079 " "	0.00021	17.5

TABLE I—*Continued.*

Experiment No.	Solution conditions.	Mols of salt added.	Average per cent deaminization.
38	25.0 cc. 0.1 N KOH		17.6
39	25.0 " 0.5 " "		23.3
40	25.0 " 1.0 " "		23.0
41	0.535 N $Ba(OH)_2$		58.2
42	0.2535 " "		49.5
43	0.1013 " "		32.6
44	0.05 " "		20.3
45	0.535 " " + 0.79 gm. $(CH_3COO)_2Pb \cdot 3H_2O$	0.0021	78.7
46	0.535 " " + 0.079 " "	0.00021	62.5
47	0.2535 " " + 0.079 " "	0.00021	55.8
48	0.2535 " " + 1.72 " $BaCl_2 \cdot 2H_2O$	0.007	56.4
49	0.1013 " " + 0.079 " $(CH_3COO)_2Pb \cdot 3H_2O$	0.00021	39.6
50	0.1013 " " + 1.22 " $BaCl_2 \cdot 2H_2O$	0.005	36.4
51	0.05 " " + 0.079 " $(CH_3COO)_2Pb \cdot 3H_2O$	0.00021	21.2
52	0.05 " " + 0.31 " $BaCl_2 \cdot 2H_2O$	0.0013	18.3
53	0.044 " $Ca(OH)_2$		24.2
54	0.044 " " + 0.079 gm. $(CH_3COO)_2Pb \cdot 3H_2O$	0.00021	29.8
55	0.044 " " + 1.3 gm. $CaC_4H_4O_6 \cdot 4H_2O$	0.005	39.8
56	0.25 gm. cystine, 0.08 gm. CaO, 50 cc. H_2O		52.1
57	0.25 " " 0.10 " " 50 " "		65.8
58	0.25 " " 0.20 " " 50 " "		84.8
59	0.25 " " 0.25 " " 50 " "		86.7
60	0.25 " " 0.30 " " 50 " "		87.9
61	0.25 " " 0.34 " " 50 " "		89.0
62	0.25 " " 1.00 " " 50 " "		88.9
63	0.25 " " 1.90 " " 50 " "		88.8
64	0.50 " " 0.30 " " 50 " "		78.8
65	0.50 " " 0.40 " " 50 " "		80.7
66	0.50 " " 0.50 " " 50 " "		82.0
67	0.50 " " 0.60 " " 50 " "		83.1
68	0.50 " " 0.70 " " 50 " "		82.5
69	0.50 " " 0.80 " " 50 " "		82.5
70	0.50 " " 0.90 " " 50 " "		81.8
71	0.25 " " 0.30 " " 25 " "		84.3
72	0.25 " " 0.10 " " 0.079 gm. $(CH_3COO)_2$-$Pb \cdot 3H_2O$, 50 cc. H_2O	0.00021	64.0
73	0.25 gm. cystine, 0.30 gm. CaO, 0.079 gm. $(CH_3COO)_2$-$Pb \cdot 3H_2O$, 50 cc. H_2O	0.00021	89.1
74	0.25 gm. cystine, 0.50 gm. CaO, 0.079 gm. $(CH_3COO)_2$-$Pb \cdot 3H_2O$, 50 cc. H_2O	0.00021	87.9

TABLE I—*Concluded.*

Experiment No.	Solution conditions.	Mols of salt added.	Average per cent deaminization.
75	0.50 gm. cystine, 0.50 gm. CaO, 0.079 gm. $(CH_3COO)_2$-$Pb \cdot 3H_2O$, 50 cc. H_2O..............................	0.00021	82.2
76	0.25 gm. cystine, 10.0 gm. $Ba(OH)_2 \cdot 8H_2O$, 50 cc. H_2O...		60.2
77	0.25 " " 5.0 " " 50 " " ...		55.3
78	0.25 " " 6.0 " MgO, 50 cc. H_2O...........		12.5
79	0.25 " " 2.0 " Ag_2O (dry), 50 cc. H_2O....		1.5
80	0.25 " " 2.0 " " (moist), 50 " "		0.5
81	0.25 " " three 1 cc. portions of 4.0 N NaOH added 1 hr. apart..................................		16.0
82	0.25 gm. cystine, six 0.5 cc. portions of 4.0 N NaOH added 45 min. apart..............................		19.6

tiveness of suspensions of $Ca(OH)_2$ in different degrees of excess was investigated (Table I, Experiments 56 to 75). The calcium oxide used was freshly ignited and as free as possible from hydroxide and carbonate. It will be seen that as we raise the proportion of calcium oxide past the limit of solubility of the hydroxide the percentage deaminization increases and reaches a maximum at about 89 per cent (in Experiments 56 to 63) with about 0.3 gm. of CaO to 0.25 gm. of cystine—a molar ratio of about 1 of cystine to 5 of CaO. Higher proportions of CaO do not raise this maximum nor has the addition of lead any effect, although below the 1:5 molar ratio, lead salts cause an increase (Table I, Experiments 72 to 74). To determine whether double the amount of cystine required double the amount of calcium oxide to reach this maximum Experiments 64 to 70 were run. However, the plateau of the curve is reached at about 0.50 gm. of CaO (instead of at 0.60) and the percentage deaminization obtained at this plateau is also somewhat lower. The influence of the total volume of the solution is shown by Experiment 71.

Suspensions of other bases were also investigated (Table I, Experiments 76 to 80). Barium hydroxide is less effective than calcium, magnesium oxide is very ineffective, and silver oxide, even when freshly prepared, causes practically no decomposition.

Acting on the hypothesis that low concentrations of alkali, fed from a reservoir, might explain the high results with calcium oxide, two experiments (Experiments 81 and 82) were made in which

TABLE II.

Percentage Deaminization of Cystine at 100° for Periods of Time Longer than 5 Hours.

Experiment No.	Solution conditions.	Time.	Deaminization.
		hrs.	*per cent*
1	0.25 gm. cystine, 0.30 gm. CaO, 50 cc. H_2O.	5	88.1
		5	0.6
		3	0.3
Total			89.0
2	0.25 gm. cystine, 0.20 gm. CaO, 50 cc. H_2O.	5	85.9
		5	1.2
		3	0.3
Total			87.4
3	0.25 gm. cystine, 0.10 gm. CaO, 50 cc. H_2O.	5	64.6
		5	7.6
		3	1.5
Total			73.7
4	0.5 gm. cystine, 0.20 gm. CaO, 50 cc. H_2O.	5	71.4
		5	4.9
		5	3.6
		5	1.7
Total			81.6
5	0.5 gm. cystine, 0.10 gm. CaO, 50 cc. H_2O.	5	21.9
		5	12.7
		5	10.2
		5	9.6
		5	7.1
Total			61.5

sodium hydroxide was introduced in smaller portions at regular intervals throughout the deaminization. As will be noted, the results indicated no advantage.

The sharp maximum of deaminization obtained with suspensions of calcium hydroxide made it of interest to determine the effects of duration of heating, both at and below the optimum proportion of calcium oxide. These experiments were prompted by the thought that the influence of various proportions of calcium oxide might bear entirely on the speed with which a final reaction was attained. Hence the data in Table II showing the result of successive periods of heating on the same solution are reported.

The results in Table II indicate that the effect of the calcium oxide is at least partly concerned with the speed of the reaction. In Experiment 1 the optimum amount of calcium oxide produced an almost complete reaction in the initial period of 5 hours heating with but little subsequent increase in the ammonia evolved. Experiments 2 and 3 show how the reaction is decelerated when smaller amounts of calcium oxide are used but they also indicate that the final figures for percentage deaminization are lower than the maximum. Experiments 4 and 5 with 0.50 gm. of cystine demonstrate the same point. These results would seem to indicate a possible stoichiometric relation between calcium and cystine in this reaction.

The ease of reactivity of cystine with lime is also of interest in connection with the method described by Van Slyke (4) for the determination of ammonia (amide nitrogen) in protein hydrolysates. Van Slyke states that no decomposition of the cystine takes place when treated with lime in a bath at 45–50° and distilled *in vacuo* for $\frac{1}{2}$ hour. In the light of the results described above it appeared worth while to investigate briefly the effect of these conditions. A parallel experiment confirmed the statement of Van Slyke after which a longer run was made in a 38° air bath. 0.50 gm. of cystine, 0.6 gm. of CaO, and 100 cc. of water were placed in a flask and aerated by means of a slow stream of air into successive flasks, each containing a measured amount of standard acid. No ammonia was found to penetrate to the second flask in any case. 17 hours of such aeration gave an amount of ammonia corresponding to 1.3 per cent deaminization; 25 hours more gave an additional 2.3 per cent while 2 weeks of steady aeration brought the percentage deaminization up to a total of about 50 per cent. The temperature of 38° is probably not much below that attained in the solution by Van Slyke's conditions so that it can safely be concluded

that no appreciable amount of cystine is decomposed under the conditions he describes. The use of air in these experiments is justified by the fact that in a number of deaminization experiments with lime at 100° it was found to give results identical with those of pure nitrogen.

Since one phase of the alkaline decomposition of cystine involves partial reduction of cysteine (1) the extent of deaminization of cysteine becomes of interest. In common with most monoamino acids, cysteine would not be expected to evolve ammonia readily and because of the extreme ease with which it is reoxidized to the easily deaminized cystine any positive results with cysteine should be viewed with considerable caution. Nevertheless a series of deaminization experiments was run with cysteine prepared by electrolytic reduction. The electrolysis was conducted in absence of air until the product showed a specific rotation of +9 to +10 (5). Samples of the acid solution of cysteine hydrochloride were then transferred without contact with air to the deaminization apparatus, the amount of sodium hydroxide requisite to produce a solution of 0.25 gm. of cysteine in 50 cc. of 2.0 M NaOH was added, and the deaminization carried out for 5 hours while sweeping with a current of O_2-free nitrogen. A number of such runs were made and in spite of all possible precautions to prevent access of air slight but definite evolution of ammonia was always observed. The percentage deaminization figures obtained varied between 3 and 5 per cent. In two cases a tube of partly neutralized cysteine solution (about pH 7) was inserted in the nitrogen train before the alkaline solution. After the experiment this solution was still dextrorotatory, thus indicating the absence of any oxygen in the train. These results make it appear possible that pure cysteine undergoes a small amount of deaminization under conditions similar to those employed with cystine. However, the conclusion reached by Marriott (3) that cysteine is more rapidly decomposed than cystine is not confirmed. Since Marriott accomplished his reduction by addition of sodium sulfide it would seem likely that this reagent was, by some other mechanism, responsible for the increase in ammonia evolution which he observed.

SUMMARY AND CONCLUSIONS.

Further studies on the deaminization of cystine in alkaline solution indicate that lead salts, when added to the alkaline solution, are more effective in increasing the extent of deaminization than any others examined. The extent of deaminization of cystine in solutions of various bases was studied, together with the effect of the addition of a variety of different salts.

Of the bases used in this reaction, suspensions of calcium hydroxide produce the greatest amount of deaminization. The attainment of a maximum percentage of deaminization for any constant amount of cystine used requires the presence of considerably more calcium hydroxide than that corresponding to a saturated solution.

Increase in calcium hydroxide over this optimum amount produces no higher degree of deaminization.

Addition of lead salts to the cystine solution containing the optimum amount of calcium hydroxide causes no increase in percentage deaminization; below this optimum proportion lead salts cause a marked increase.

Experiments on pure cysteine under conditions similar to those used for cystine, indicate a percentage deamination of between 3 and 5 per cent.

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SOME CHEMICAL INVESTIGATIONS OF EMBRYONIC METABOLISM.

V. THE TYROSINE, TRYPTOPHANE, CYSTINE, CYSTEINE, AND URIC ACID CONTENT OF THE DEVELOPING HEN'S EGG.

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Some incomplete studies of the tyrosine, tryptophane, and cystine content of the developing hen's egg have been made by Sendju (1). The tyrosine was determined by the bromination method of Fürth and Fleischmann (2), the tryptophane by the colorimetric method of Voisenet (3) as applied quantitatively by Fürth and Nobel (4), and the cystine was determined by the iodine method of Okuda (5). This method is based on the fact that when cystine is reduced to cysteine and treated under standard conditions with an excess of potassium iodide in acid solution a definite amount of standard potassium iodate solution is required to liberate the iodine from the potassium iodide, which in turn reacts with the cysteine. The end-point is determined by the appearance of a yellow color which indicates the presence of an excess of iodine. A modification of the method has been used by Okuda (6) for the determination of cystine and cysteine in various tissues among which were egg yolk and egg white.

Cystine has also been determined in the developing hen's egg by Plimmer and Lowndes (7) and by Calvery (8) by the nitrogen distribution method of Van Slyke. The uric acid has been studied most thoroughly by Fiske and Boyden (9) and by Needham (10). Fiske and Boyden varied their technique but the final estimation was made colorimetrically while Needham used the method of Benedict and Franke (11) in the early stages but precipitated the uric acid, after the 11th day, with saturated ammonium chloride. The values obtained by Fiske and Boyden are more than twice as large as those obtained by Needham.

The observations of Sendju (1) were not made daily on a complete series of developing eggs and the methods for tyrosine and tryptophane which he used may be subject to criticism. In view of these facts and the fact that cysteine has not been determined at all it seemed advisable to repeat the determinations on a complete

series of eggs, different methods for comparison being used, and also to determine the cysteine content in a new series of developing eggs. In this investigation the colorimetric method of Folin and Ciocalteu (12) was used for the determination of tyrosine and tryptophane. The cystine, "cysteine," and "uric acid" were determined by the method of Folin and Marenzi (13) while the cystine and cysteine were also estimated by the modification of the method of Okuda (6) for the prevention of oxidation during hydrolysis, as further modified by Teruuchi and Okabe (14).

EXPERIMENTAL.

Two series of investigations were made. In the first series tyrosine, tryptophane, cystine, and uric acid were estimated on part of the dry material (the preparation of which has previously been described) which was used in the previous study by the Van Slyke nitrogen distribution method (8) and for the estimation of the basic amino acids (15). The tryptophane was determined by the two methods of Folin and Ciocalteu without any modification, while the tyrosine was estimated only by their first method (Millon's). Their second method (with the phenol reagent) could not be used because of clouding which could not be overcome.

The cystine was determined by the method of Folin and Marenzi (13) with the following modifications. First it was necessary to centrifuge the solution after the color developed in order to remove the precipitate which was always present. This necessitated a longer period of waiting before reading the color than is recommended and may detract from the accuracy of the method although no color is carried down with the precipitate. In the second place it was necessary to determine a "blank;" that is, the color produced when distilled water was used in the unknown instead of the reducing agent (sulfite). This blank produced in the absence of the reducing agent was called "uric acid" in this investigation. This figure was subtracted from the figure obtained in the presence of the sulfite and was called total cystine. The determinations of cystine and uric acid in the first series of eggs were so nearly the same as those of the second series that they have not been included in the tables. In order to conserve space values for some of the significant days have been recorded in Tables I and II although complete analyses were made every day throughout the entire period of development.

A standard solution of uric acid both with and without the reducing agent (sulfite) was compared with a standard solution of cystine and it was found that 1 mg. of uric acid produced as much color as 1.408 mg. of cystine. Consequently this factor was used in making the calculations for "uric acid." The sulfite had no influence on the color produced by uric acid.

In order to determine the cysteine content of tissue it is necessary that fresh material be used and precautions be taken so that the cysteine is not oxidized to cystine during hydrolysis. For these reasons a new series of freshly incubated eggs was necessary. Eggs from thoroughbred White Leghorn hens were incubated at 38° ± 0.5° in a circulating atmosphere which was 84 per cent saturated with moisture. The eggs were turned twice daily. Five eggs were removed for investigation each morning at a definite hour. After being weighed and examined to determine the age of the embryo the contents of the shells of five eggs were poured into 1500 cc. of boiling absolute alcohol. They were stirred thoroughly for a few minutes while the alcohol was again brought to the boiling point on a hot plate and immediately filtered. The coagulum was extracted three times with 300 cc. portions of ethyl ether. In the later stages it was necessary to cut the embryos into small pieces. That the lipids were practically completely removed by this procedure and only very small amounts of non-lipid material was demonstrated by the fact that when the ether and alcohol were removed by distillation under reduced pressure and the residue extracted with petroleum ether only a very small amount of insoluble (non-lipid) material remained while the weight of the material soluble in petroleum ether is greater than that reported by most investigators.[1]

Difficulty was experienced when deproteinization was attempted by the method recommended by Okuda (5) so that the above procedure was substituted. Its chief advantages are its complete precipitation of proteins, and the removal of the large amounts of lipids, as well as the rapidity with which it can be executed. The extracted material, including the shells, was immediately placed in a liter round bottom flask containing 500 cc. of 25 per cent hydro-

[1] The information concerning the alcohol- and ether-soluble material was obtained from Dr. H. C. Eckstein of this department who is investigating this fraction.

chloric acid and hydrolyzed for 10 or 12 hours, while a continuous stream of carbon dioxide was passed through the solution. The hydrolysate was analyzed by the Teruuchi and Okabe (14) modification of the Okuda (5) method. The modification possesses several advantages. (1) The time is considerably shortened since a maximum yield of cystine can be obtained by hydrolyzing for only 7 to 10 hours, and concentration of the hydrolysate is not necessary. (2) The decolorization by charcoal is omitted and as a result there is a no cystine or cysteine lost by removal with the charcoal. (3) The permanent blue color obtained when starch is used as an indicator is much more definite than the slightly yellow color produced when an excess of iodine is present in the solution, especially when the solution is slightly colored before the titration is begun. (4) Finally it has the advantage that the temperature corrections of Okuda (5) are not necessary since a standard solution of cystine is always reduced and titrated at the same time the unknown is titrated.

The acid concentration of the hydrolysate was adjusted to 2 per cent and the volume to 1 liter. The cysteine was determined in a sample of this solution without reduction and the cystine in a sample after reduction with zinc.

The cystine, "cysteine," and "uric acid" were also determined in the second series of eggs by the method of Folin and Marenzi (13). In order to determine the three above substances by this method the following procedure was used. Cystine was used as the standard in the usual manner and the color produced by a definite volume of the unknown, with distilled water instead of sulfite, was compared with the standard. This was calculated as cystine but was called "cysteine" plus "uric acid."

A definite volume, usually 100 cc., of the hydrolysate was then made faintly alkaline with ammonia and aerated for 5 hours or boiled for a few minutes (procedures which were shown to convert cysteine to cystine). The volume was again adjusted to 100 cc. and the acid concentration to 2 per cent and the color produced by a definite volume of this solution without sulfite was compared with that produced by a standard cystine solution prepared in the usual manner. This was calculated as cystine but was called "uric acid." (In order to get the true uric acid value the conversion factor previously mentioned was used.)

The difference between the value obtained by the first procedure, the "cysteine" plus "uric acid," and that obtained by the second procedure, the "uric acid," gave the "cysteine" content of the solution. A third determination was made on a definite volume of the solution by the addition of the sulfite exactly as recommended by Folin and Marenzi (13) and a value was obtained and calculated as cystine which should have included cystine, "cysteine," "uric acid," and any other substance which would produce a color under these conditions. From this total value the true cystine content of the solution may be obtained by subtraction of the value for "cysteine" plus "uric acid" obtained by the first procedure. The sum of the "cysteine" and cystine can be obtained by subtraction of the value calculated as cystine and called "uric acid" obtained by the second procedure. These values were all calculated in terms of mg. per egg and are recorded in Table II.

TABLE I.

Tyrosine and Tryptophane Content of the Developing Hen's Egg Determined by the Method of Folin and Ciocalteu (12).

Period of incubation.	Dry weight of five eggs.	Total N in five eggs.	Tyrosine (Millon's) per egg.	Tryptophane.	
				First method per egg.	Second method per egg.
days	*gm.*	*gm.*	*mg.*	*mg.*	*mg.*
Fresh egg.	62	1.14	322	119	116
6	66	1.07	316	130	121
11	63	1.20	284	124	115
16	62	1.12	286	122	106
19	59	1.11	276	114	100
Hatched chick.	60	0.98	212	134	108

DISCUSSION.

Although the tyrosine values reported by Sendju (1) are higher than those recorded in Table I, they show the same general tendency to decline during the period of incubation. His values for tryptophane are also higher in the beginning of the period of incubation than those obtained in this investigation and neither of the methods of Folin and Ciocalteu (12) shows a decrease. The

method of Fürth and Nobel (4) as used by Sendju (1) showed a marked drop on the 3rd day which is coincident with the first formation of blood pigments. Sendju believes there is a relationship between this sudden drop in tryptophane content and the appearance of the blood pigments. His explanation is hardly adequate since there is a synthesis of large amounts of blood pigments each day until the 10th day at least (16) and on the 10th day his values for tryptophane are higher than on the 3rd day. He also believes that there is a relationship between the sudden drop of tryptophane on the 14th day and the appearance of bile pigments. Again the bile pigments do not all appear at once, but are synthesized over a period of several days (16). The tryptophane content as determined in this investigation shows no such changes.[2] It cannot be definitely stated that the values reported as "uric acid" in this investigation represent the true uric acid content although they do check very closely with those obtained by Needham (10). However, they are *much smaller* than the values reported by Fiske and Boyden (9). They were not checked by other methods, but were obtained as a blank in order to determine true cystine values. There is good agreement between the values obtained in the two series of eggs.

The cystine values as determined by the iodine method of Okuda (5) check each other very closely in the two series of eggs only one of which is reported here and also agree very well with the values reported by Sendju (1) in the early stages of development. Near the end of the period, however, the values reported are much lower than those reported by Sendju as determined by the same method.

The method of Folin and Marenzi (13) which is a modification of the method of Folin and Looney (17) for cystine gives much higher values than the Okuda method. This may be due to interfering substances since Okuda (5) has pointed out that there are other substances present in a protein hydrolysate besides cystine that will give a color with the Folin and Looney reagent. It must be kept in mind also that in a defatted chick embryo there are a large number of substances which would not be present in a purified protein.

[2] It is possible that in the method of Folin and Ciocalteu (12) there are other substances present which react to produce color which would be calculated as tryptophane, whereas these substances might not react in the method of Fürth and Nobel (4) and this fact may account for the differences between the values obtained in the two investigations.

Since the animal organism probably does not synthesize cystine it is very difficult to explain the gradual increase in the cystine content during development as indicated by the method of Folin and Marenzi even after correction for "uric acid." However, if the values obtained by Fiske and Boyden (9) for uric acid were used in correcting the total color value produced by the Folin and Marenzi reagent there would be practically no increase indicated in the

TABLE II.

Cystine, Cysteine, and Uric Acid Content of the Developing Hen's Egg Determined by the Modified Method of Folin and Marenzi (13) and the Cystine and Cysteine Content Determined by the Modified Method of Okuda (5, 6).

Period of incubation.	Wet weight of five eggs.	Total N in five eggs.	Values obtained by the Okuda method. Cystine plus cysteine per egg.	Cysteine per egg.	Cystine per egg.	Ratio $\frac{\text{cystine}}{\text{cysteine}}$.	Values obtained by the Folin and Marenzi method. Total color calculated as cystine per egg.	Total color less uric acid per egg.	Cysteine per egg.	Cystine per egg.	Uric acid per egg.	Difference between values for cysteine + cystine by Folin and Marenzi's and Okuda's methods.
	gm.	*gm.*	*mg.*	*mg.*	*mg.*		*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Fresh egg.	300	5.040	149	21	127	5.9	237	237	19	217	None.	88
4	315	5.544	159	38	121	3.1	234	233	40	193	Trace.	74
8	306	5.728	142	67	75	1.1	226	226	71	155	"	84
12	280	5.096	120	42	78	1.8	279	274	41	233	4.4	153
15	265	5.082	116	37	78	2.0	289	274	35	239	10.9	157
20	265	5.380	90	61	28	0.4	343	299	63	236	30.9	209
Hatched chick.	255	5.152	76	42	34	0.8	352	314	41	273	33.5	238

cystine content. However there would be no decrease as shown by the Okuda method. There was no decrease indicated by the Van Slyke nitrogen distribution method in the determinations of either Calvery (8) or Plimmer and Lowndes (7) in which an average content of about 140 mg. per egg was indicated. It seems that the only explanation of the increase indicated by the Folin and Marenzi method is the synthesis of interfering substances by the embryo during development. Lewis and Nicolet (18) and Funk and Macallum (19) have studied a large number of substances

among which were some purine and pyrimidine derivatives which react with the phosphotungstic acid reagents and since these types of compounds are synthesized by the embryo they may account for the extra color produced.

The "cysteine" values as determined by both methods on the second series of eggs (cysteine could not be determined on the first series since no precautions were taken to prevent its oxidation to cystine) showed very close agreement between the two methods but marked variations during the period of development. There is a striking difference between the total cysteine content and the ratio of cystine to cysteine in the fresh egg when compared with the later stages of development. Okuda (6) was probably the first to present quantitative evidence to support the theory that the more active the tissue, biologically, the greater the ratio of cysteine to cystine. Such a theory is well supported by the data recorded in Table II for cysteine, since, during the later stages the cysteine content of the developing hen's egg is, in some cases, three times as high as in the unincubated egg. Also the ratio of cystine to cysteine at the beginning is nearly 6 while on the 20th day just at the time of hatching the ratio is 0.46.

The values for cysteine show two peaks and at the same points the ratios of cystine to cysteine show a drop. One of the peaks for cysteine comes on the 8th day after which there is a gradual decline to the 15th day and then a rise to the second peak which appears on the 20th day. The explanation of this rise and fall and second rise is very difficult. According to Huxley (20) there are three fundamentally different processes, essentially consecutive, though occasionally overlapping, to be distinguished in the developing amphibian egg up to the time of hatching. First there is division and rearrangement of the material and structure present at the beginning, then there is non-functional differentiation including determination and differentiation of the primordia of organs. Finally, there is functional differentiation. Now it may be possible that there are three such periods during the development of the hen's egg. There is division and rearrangement of preexisting material in the developing hen's egg according to the embryologist up to about the 8th or 9th day, when the "anlage" of all the organs has been determined, then there is a period of growth and adjustment with very little function of the organs up to about the 15th day, and finally during the 3rd week there is a marked increase in chemical

activity as indicated by a large increase in heat production and carbon dioxide output. It may be possible to associate certain chemical changes during development with these different periods. The first peak in the cysteine values is reached at exactly the end of the first period indicated above. The fall in the cysteine content corresponds to the second period or the period of growth with very little function while the second rise corresponds to the third period, the period of greatest chemical activity, and reaches a peak at the time the chick is fully developed.

The changes in the tyrosine content show some correspondence to the periods of differentiation, growth, and function of the embryo mentioned above but the relationship is not nearly so close as the changes in the cysteine content. There is a fall at first then it remains almost constant through the middle of the period, then there is a sudden decrease at the time of hatching. Needham (10) has shown that the peak in the protein metabolism of the developing hen's egg is reached about the 8th day and this would correspond to the first peak in cysteine content and the drop in tyrosine content but no such peak has been reported for protein near the end of the period although it is during this last period that nearly all the fat is burned.

If the cysteine content is directly connected with oxidative processes a rise in the cysteine would be expected and a decrease at any period would be unexpected since the carbon dioxide and heat output increase throughout the entire period of development after the 4th day (21). It should also possibly be related to the glutathione content but Yaoi (22) and Murray (23) have found that there is an increase in glutathione content up to about the 14th day and thereafter a decrease, consequently there seems to be no direct relationship.

SUMMARY.

1. The tyrosine, tryptophane, cystine, cysteine, and uric acid contents of the developing hen's egg were determined on a complete series of eggs, five eggs being used each day throughout the entire period of development.

2. The tyrosine content decreases but the tryptophane shows no decrease by either of the two methods used which is in contrast with data reported by Sendju (1), which showed a marked decrease in both tryptophane and tyrosine.

3. The cystine content was determined on two series of eggs by two different methods. The values obtained by the Okuda (5) method agree very well for the two series and with values previously reported by Sendju (1) for the early stages but not for the later stages of development. The values obtained by a modification of the Folin and Marenzi (13) method agree very closely for the two series of eggs but do not agree at all with the values obtained by the Okuda method. They are much higher at the beginning of the period and show an increase throughout the entire period whereas the cystine content as determined by the Okuda method shows a marked decrease.

4. The cysteine content was determined by two methods and the values agree very closely. It reaches a peak on the 8th day, thereafter it declines to a minimal value on the 15 day, and then reaches a second peak at the time of hatching.

5. The relationship of the cysteine content to the three periods of development, to the glutathione content, and to the activity of the tissue biologically is discussed.

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STUDIES ON CRYSTALLINE INSULIN.*

IX. THE ADSORPTION OF INSULIN ON CHARCOAL.

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It was thought desirable to repeat the work of Dingemanse who claims to have been successful in obtaining an insulin preparation with an activity of about 150 international units per mg. (1). Du Vigneaud, Geiling, and Eddy (2) who studied the adsorption of crystalline insulin on charcoal found that the product obtained after adsorption was not more active than the material they started with and that it also could be again obtained in crystalline form. These investigators used norit (Eastman Kodak Company) as an adsorbent. Dingemanse, however, states in a report which she kindly sent us that only with a certain Dutch preparation—medicinal supranorit obtained from the Noritmaatschappij Ltd.—could positive results be obtained by her and that Merck's charcoal, for instance, was useless. This information, however, was not available to du Vigneaud, Geiling, and Eddy.

I. Adsorption of a Pyridine Precipitate on Charcoal (Supranorit).

In the beginning of the past year the adsorption of insulin on charcoal with the preparation specified by Dingemanse had already been repeated in this laboratory. The procedure employed by us may be shortly outlined: 200 mg. of the pyridine precipitate obtained from the concentrated commercial solution of insulin (Squibb) by adding 13.5 per cent pyridine and evaluated at about 20 units per mg. were dissolved in 150 cc. of 0.01 N HCl, to the clear solution were added 400 mg. of charcoal (supranorit Dutch preparation), and the whole was shaken for 1 hour. After filtration the charcoal was washed with absolute alcohol and ether.

* An investigation carried out under a grant from the Carnegie Corporation of New York.

It was found that by neutralizing the filtrate a comparatively large precipitate was formed, thus indicating that not all the insulin had been adsorbed; the precipitate was not evaluated, but contained apparently all the impurities. The dried charcoal powder was ground up with 3 cc. of 90 per cent phenol in a mortar, let stand for about 10 minutes, and then filtered with mild suction through a hardened filter. The charcoal was again mixed well twice with 3 cc. of 90 per cent phenol. The combined phenolic filtrates which contain the insulin were poured into 30 times their volume of water and stirred well until all the phenol had been dissolved in water. The insulin which flocks out was centrifuged off, and washed with absolute alcohol and ether. This preparation (Preparation A) was compared with crystalline insulin which had been evaluated at about 24 units per mg., according to the same method given in a previous paper from this laboratory (3). As can be seen from Table I, Experiments 1 and 2, this preparation was not more active than crystalline insulin but had about the same strength as crystalline insulin. The increased activity of the adsorbed product over that of the preparation started from shows that purification of insulin can be effected in this way, as has also been shown by Moloney and Findlay (4). The figures given in Table I are the average values obtained with the number of animals indicated. The animals were used in groups of four on different days according to the procedure outlined in a previous communication (3).

We thought it advisable to find out whether perhaps by a second adsorption of this product (Preparation A) on charcoal a stronger preparation than crystalline insulin could be obtained. In the final step of the purification of the Dutch insulin preparation (organon) M/15 disodium hydrogen phosphate solution was used as a solvent by Dingemanse. She also stated that the products obtained in the final step of purification are rather unstable and have to be injected the same day when prepared in order to obtain the high values of the activity. We, therefore, also employed M/15 disodium hydrogen phosphate solution as a solvent and all preparations were standardized against crystalline insulin the same day immediately after having been secured. The procedure of adsorption was similar to that outlined above. We therefore give only the amounts used: 20 mg. of Preparation A, dissolved

TABLE I.

Experiment No.	Preparation.	No. of rabbits.	Average weight.	Dose per kilo.	Average of blood sugar, mg. per 100 cc. Normal.	$1\frac{1}{2}$ hrs.	3 hrs.	5 hrs.	Convulsions.
			kg.	*mg.*					
1	Crystalline insulin dissolved in M/15 Na_2HPO_4.	12	2.05	1:40	109	59	86	104	2
2	Squibb's pyridine precipitate. First adsorption on charcoal. Dissolved in M/15 Na_2HPO_4.	12	2.07	1:40	104	61	81	101	4
3	Crystalline insulin dissolved in M/15 Na_2HPO_4.	12	2.19	1:40	114	57	80	95	3
4	Squibb's pyridine precipitate. Second adsorption on charcoal. Dissolved in M/15 Na_2HPO_4.	12	2.12	1:40	122	67	92	100	None.
				Dose per animal.					
5	Crystalline insulin kept in solution overnight. Dissolved in M/15 Na_2HPO_4.	12	1.91	1:125	109	74			
6	Dutch insulin (preparation of Dr. Dingemanse) kept in solution overnight. Dissolved in M/15 Na_2HPO_4.	12	1.80	1:125	115	97			
7	Crystalline insulin kept in solution overnight. Dissolved in M/15 Na_2HPO_4.	4	1.84	1:65	113	54			
		4	1.97	1:125	117	73			
8	Crystalline insulin adsorbed on charcoal kept in solution overnight. Dissolved in M/15 Na_2HPO_4.	3	1.80	1:65	114	60			
		4	2.11	1:125	101	69			

TABLE I—*Concluded.*

Experiment No.	Preparation.	No. of rabbits.	Average weight.	Dose per kilo.	Average of blood sugar, mg. per 100 cc.				Convulsions.
					Normal.	$1\frac{1}{2}$ hrs.	3 hrs.	5 hrs.	
			kg.	*mg.*					
9	Crystalline insulin dissolved in M/15 Na_2HPO_4.	12	2.02	1:80	112	52	83		3
10	Pig insulin adsorbed on charcoal. Dissolved in M/15 Na_2HPO_4.	12	2.04	1:80	110	48	64		
11	Crystalline insulin dissolved in M/15 Na_2HPO_4.	4	1.92	1:80	99	35	55		1
12	Pig insulin adsorbed on charcoal kept in solution overnight. Dissolved in M/15 Na_2HPO_4.	4	1.94	1:80	108	59	79		

in 15 cc. of M/15 disodium hydrogen phosphate, were shaken with 20 mg. of charcoal (supranorit, Dutch brand). Charcoal powder was extracted three times with 1 cc. of 90 per cent phenol.

As can be seen from Experiments 3 and 4 (Table I) no increase in activity could be observed; the preparation obtained after the second adsorption had the same activity as the material started from and was found to be about as active as crystalline insulin.

Our findings led us to believe that we had accumulated enough evidence to show that it was not possible under the conditions described above to obtain a more active preparation than crystalline insulin, at least not from the Squibb's product. Later, however, one of us was informed by Doctor Dingemanse that not all grades of the supranorit (Dutch preparation) were equally suitable for this purpose. Through the kindness of Doctor Dingemanse we came into possession of some charcoal and also some insulin (organon) which according to Doctor Dingemanse had been used by her with success.

II. Adsorption of Crystalline Insulin on Charcoal.

First of all we thought it of importance to see if by adsorption of crystalline insulin on this particular grade of charcoal a more active product could be obtained. On account of the stated instability of the more highly active products, the adsorbed insulin was standardized against crystalline insulin on the very day of its preparation. M/15 disodium hydrogen phosphate was again used as a solvent. Procedure and amounts employed were as given under the description of the adsorption of Preparation A on charcoal.

As can be seen from Tables II and III no increase of activity of the adsorbed crystalline insulin over that of the crystalline insulin started from could be found. It seems, therefore, that it is not possible to obtain a more active preparation from crystalline insulin even with the special grade of charcoal used by Dingemanse.

Some crystalline insulin was sent in the fall of 1929 to Doctor Dingemanse so that she might try to get a more active product from crystalline insulin. Unfortunately, we have not heard from her up to now about the outcome of her experiments. There remains, of course, still to be considered the possibility that by the process of manufacture of the Squibb's insulin the more active form of insulin, if such exists, has been destroyed or changed to a more stable but less active form. We were therefore anxious to see if we could obtain the more active preparation of Dingemanse by repeating her work as closely as possible, using the *same* charcoal and the *same* insulin preparation that she did.

III. Repetition of Work of Dingemanse.

2.5 gm. of insulin, organon, preparation of Dingemanse which had an activity of about 2.5 units per mg. were shaken with 50 cc. of 0.01 N sodium bicarbonate solution. All the material went into solution; the pH of this solution was found to be about 4.5, which explains why all was dissolved. The pH of the solution was adjusted to about 7 by adding 0.01 N sodium bicarbonate. The precipitate, which according to Dingemanse should contain nearly all the insulin whereas most of the impurities should remain dissolved, was centrifuged off, and washed with absolute alcohol and ether. About 1 gm. was obtained.

1 gm. of this purified insulin preparation was dissolved in 200 cc. of 0.01 N hydrochloric acid. The solution which was still

TABLE II.

Crystalline Beef Insulin, 1 Mg. Dissolved in 65 Cc. of M/15 Na_2HPO_4.

The actual dose (1 cc. of the preparation) contained $\frac{1}{65}$ mg. of insulin.

Date.	Rabbit No.	Weight.	Blood sugar.			
			Normal.	1 hr.	1½ hrs.	
1929		*kg.*	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*	
Oct. 9	1	2.38	122	71	82	
	2	1.40	133	56	49	Convulsions.
	3	2.44	127	67	71	
	4	1.55	105	51	44	
" 16	5	2.15	117	58	62	
	6	2.40	108	80	76	
	7	1.84	115	51	51	
	8	2.66	98	58	73	
" 11	9	1.98	94	53	33	Convulsions.
	10	1.55	87	51	46	
	11	1.85	98	62	60	
	12	2.10	92	58	53	
" 18	13	2.34	107	68	68	
	14	1.57	99	59	54	
	15	1.60	118	50	57	
	16	2.60	111	46	54	
" 14	17	2.10	115	42	57	
	18	1.60	112	55	51	
	19	2.04	112	71	78	
	20	1.58	112	50	39	
" 21	21	1.80	108	49	42	Convulsions.
	22	1.73	106	60	71	
	23	Died.				
	24	1.60	144	62	67	

acid after all the insulin had gone into solution was shaken with 2 gm. of medicinal supranorit (preparation of Dingemanse) for 1 hour. It was found that the solution gradually became neutral,

probably due to the presence of basic metallic oxides like calcium oxide in the charcoal. We therefore used more 0.01 N hydrochloric

TABLE III.

Crystalline Beef Insulin, Adsorbed on Charcoal, 1 Mg. Dissolved in 65 Cc. of M/15 Na_2HPO_4.

The actual dose (1 cc. of the preparation) contained $\frac{1}{65}$ mg. of insulin.

Date.	Rabbit No.	Weight.	Blood sugar.			
			Normal.	1 hr.	1½ hrs.	
1929		*kg.*	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*	
Oct. 16	1	2.45	103	58	67	
	2	1.78	110	58	58	
	3	2.50	98	66	71	
	4	1.60	100	53	40	
" 9	5	2.20	117	83	78	
	6	2.30	124	103	101	
	7	1.90	110	85	83	
	8	1.91	105	76	47	
" 18	9	1.97	91	25	28	
	10	1.45	109	41	55	
	11	1.80	97	55	57	
	12	2.07	90	57	55	
" 11	13	2.35	110	74	96	
	14	1.73	94	71	64	
	15	1.63	113	76	67	
	16	2.65	96	58	65	
" 21	17	2.04	133	76	73	
	18	1.54	119	62	65	
	19	2.03	119	78	73	
	20	2.20	106	65	74	
" 14	21	1.73	105	41	46	
	22	1.83	108	69	68	
	23	2.32	98	62	73	
	24	1.51	107	33		Convulsions.

acid when this experiment was repeated so that the solution was always acid. The charcoal on which the insulin was adsorbed was

filtered with mild suction, and washed with absolute alcohol and ether. It was then ground up in a mortar with 5 cc. of 90 per cent phenol, and after standing for 10 minutes was filtered through a hardened filter with mild suction, and the charcoal powder was again mixed well twice with 2 cc. of 90 per cent phenol and filtered. The combined phenolic filtrates were poured into 30 times their volume of water. With the help of stirring the phenol was

TABLE IV.

Crystalline Insulin, 1 Mg. in 125 Cc. of M/15 Na_2HPO_4.

The actual dose (1 cc. of the preparation) contained $\frac{1}{125}$ mg. of insulin.

Date.	Rabbit No.	Weight.	Blood sugar.	
			Normal.	$1\frac{1}{2}$ hrs.
1929		*kg.*	*mg. per 100 cc.*	*mg. per 100 cc.*
Nov. 7	34	1.98	95	59
	11	1.83	108	64
	15	1.67	117	69
	64	2.10	121	62
" 13	56	1.70	108	87
	11	1.92	124	74
	32	1.80	112	72
	69	1.70	107	69
" 9	60	2.00	116	75
	7	1.92	123	84
	54	1.65	130	69
" 11	17	2.05	122	74
	19	2.13	119	91
	62	1.80	120	96
	45	1.60	105	73

gradually brought into solution; the insulin was centrifuged off, and washed with absolute alcohol and ether. The insulin preparation thus obtained was adsorbed a second time on the supranorit of Dingemanse in 0.01 N hydrochloric acid solution, employing the corresponding amounts of reagents. About 100 mg. of insulin were obtained after this second adsorption from the 2.5 gm. of insulin (organon) started from. The activity of this preparation which according to Dingemanse should be at least as high as

that of crystalline insulin was not determined. This product was used as the starting material for the final step in the procedure employed by Dingemanse for the preparation of her highly active insulin. In this final step M/15 disodium hydrogen phosphate was used as a solvent. We found in agreement with Dingemanse that only a comparatively small amount of this product was soluble

TABLE V.

Dutch Insulin (Preparation of Dingemanse) Adsorbed on Charcoal, 1 Mg. in 125 Cc. of M/15 Na_2HPO_4.

The actual dose (1 cc. of the preparation) contained $\frac{1}{125}$ mg. of insulin.

Date.	Rabbit No.	Weight.	Blood sugar.		
			Normal.	1½ hours.	
1929		*kg.*	*mg. per 100 cc.*	*mg. per 100 cc.*	
Nov. 13	34	1.95	138	87	
	12	2.13	107	72	
	15	1.65	142	101	
	64	2.10	119	99	
" 7	56	1.73	101	77	
	12	2.10	100	80	
	32	1.82	100	66	
	69	1.70	96	78	
" 8	58	1.60	114	64	Died.
	2	1.65	123	86	
	6	2.27	128	109	
	3	2.20	128	95	
" 11	21	1.90	105	83	
	22	1.75	100	82	
	52	1.80	127	103	
	95	1.92	122	117	

in this solvent. We did not find out whether the insoluble part was active. The procedure employed was quite similar to the one given for Preparation A (second adsorption) and crystalline insulin.

40 mg. of the insulin preparation which had been purified by isoelectric precipitation and two adsorptions on charcoal were

mixed well with 20 cc. of M/15 disodium hydrogen phosphate. 15 minutes after standing the solution was centrifuged and the clear supernatant fluid poured off and shaken with 20 mg. of supranorit (preparation of Dingemanse) for half an hour. The charcoal powder was extracted three times with 1 cc. of 90 per cent phenol. The insulin preparation thus obtained was standardized against crystalline insulin the same day, immediately after it was obtained. According to Dingemanse this preparation should be much more active than crystalline insulin. Tables IV and V clearly show, however, that we were not able at any time to secure a preparation which was more active than crystalline insulin; the activity was about the same as that of crystalline insulin. The experiment just outlined has been carried out independently by two investigators in this laboratory, with the same results.

Dingemanse found that this final product is rather unstable and loses most of its activity whether kept in solution or in dry form. We could confirm this finding of Dingemanse. As can be seen from Experiments 5 and 6 in Table I the Dutch insulin preparation has lost a great part of its activity when let stand in solution overnight. Crystalline insulin will lose no appreciable amount of its activity under these conditions. In the third series of these experiments when we used an insulin preparation which had been kept in a desiccator for several days, the result was the same.

We were interested to see if crystalline beef insulin would become also more unstable by adsorption on charcoal. Crystalline insulin was therefore adsorbed on charcoal, and the obtained product kept in M/15 disodium hydrogen phosphate solution overnight, and was then compared with crystalline insulin. As Experiments 7 and 8 in Table I indicate, no loss in activity could be observed.

IV. Adsorption of Pig Insulin on Charcoal.

We have been informed that the Dutch insulin preparation (organon) contains about 70 per cent of pig insulin. We thought that perhaps pig insulin might give a more active preparation than crystalline beef insulin. For this reason we studied the adsorption of pig insulin on charcoal. The preparation of pig

insulin,[1] from which we started, was evaluated and found to be about as active as crystalline insulin. The adsorption on charcoal was carried out similarly to that previously outlined. Experiments 9 and 10 (Table I) show that the adsorbed preparation was not more active than crystalline beef insulin but had about the same activity.

We also found that the adsorbed pig insulin does not lose any appreciable amount of its activity by letting it stand in M/15 disodium hydrogen phosphate solution overnight, as Experiments 11 and 12 demonstrate.

CONCLUSIONS.

The experiments outlined in this paper clearly indicate that we have not been successful in obtaining a preparation more active than crystalline beef insulin. We therefore cannot substantiate the reported finding by Dingemanse of an insulin preparation which according to her should contain about 150 international units per mg. We are not in a position to explain the results of Dingemanse. Unfortunately, Dingemanse did not compare her highly active preparation with a standard preparation, which in our minds is quite important in determining the absolute value for the activity of an insulin preparation. As can be seen in Experiments 9 and 11 (Table I) and in Table II, from the figures obtained with crystalline insulin, we have obtained sometimes quite a lowering of blood sugar with a comparatively small dosage of crystalline insulin. According to the method of evaluation employed by Dingemanse, these figures would give a much higher value for the activity of crystalline insulin than is obtained on a comparative basis.

SUMMARY.

Starting with various insulin preparations—pyridine precipitate, crystalline insulin, and pig insulin—and submitting them to the adsorption on charcoal according to Dingemanse, using the same grade as employed by Dingemanse, we have completely failed in obtaining a product more active than crystalline insulin.

[1] The material was kindly prepared for us by E. R. Squibb and Sons, New Brunswick, New Jersey, and we wish to express our best thanks to them.

In repeating the work of Dingemanse using the same insulin preparation and charcoal as employed by Dingemanse, we were not able to secure a preparation more active than crystalline insulin. We found in agreement with Dingemanse that the final preparation obtained from organon is rather unstable. The results of our experiments do not substantiate the claim of Dingemanse to be able to obtain an insulin preparation which is more active than crystalline insulin.

Addendum.—When this paper was in press we received an answer from Doctor Dingemanse to whom we had sent a copy of the manuscript. According to this personal communication one has to use a certain amount of supranorit for a given quantity of insulin and with different insulin preparations one always has to find out first the exact amount of supranorit to be used in order to obtain positive results. With the amount of supranorit employed in her recent experiment with crystalline insulin Doctor Dingemanse was not able to secure a more active preparation than the crystalline insulin itself, but she thinks that this negative result may be due to the fact that the exact amount of charcoal necessary for the success of the experiment had not first been ascertained. Doctor Dingemanse also informed us that she has lately compared the highly active preparations obtained by her with crystalline insulin and again reports that her preparations have a higher activity. It seems to us that the experimental conditions which should give positive results have to be put on a much sounder basis before her results can generally be accepted. We think, however, that one has to maintain an open mind on the possibility of preparing from pancreatic extract a product more active than crystalline insulin. Although our results in repeating the work of Dingemanse as closely as possible have been negative in the sense that we were not able to secure a preparation more active than crystalline insulin, we present our findings with the hope of inducing other laboratories to repeat the work. Doctor Dingemanse also informs us that workers in other laboratories have been unable to repeat her work. We should like to express here again our great appreciation for the cooperation given us by Doctor Dingemanse.

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SOME REDOX INDICATORS.

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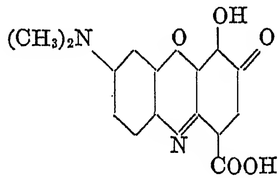
The colorimetric determination of oxidation-reduction potentials by means of dyestuff indicators has been placed on a sound theoretical foundation by Clark and his associates (1). Some more indicators have been recently measured by Rapkine, Struyk, and Wurmser (2), furthermore by Vellinger (3). The practical application of this principle can be improved upon only by finding new indicators, in the first place to enlarge the range of redox potential accessible to a colorimetric determination especially towards the negative (hydrogen) side of the potential scale, and secondly to duplicate the dyes now available with others covering the same potential range, but of different chemical constitution. The agreement of a potential determined by several indicators of different chemical constitution is the best evidence for the reliability of the indicator method.

In the present paper three dyestuffs are to be described which may be useful for the indicator method. All three have been known for a long time but were never used for any scientific purpose. They form entirely reversible redox systems and give true homogeneous non-colloidal solutions in the ordinary buffers in a concentration sufficient for any colorimetric use.[1] These dyes turned out to be practically pure as delivered from the factory in so

[1] The authors are indebted to the I. G. Aktiengesellschaft Farbenindustrie, Germany, for these dyes. They were selected from a large collection of dyes placed at our disposal according to our suggestions from the stock of the scientific laboratories of this company. We are especially indebted to the liberality of Dr. H. Benda and Dr. I. Peiser. The dyestuffs will be distributed by the Winthrop Chemical Company, Incorporated, 170 Varick Street, New York, N. Y. These will be in accurate agreement with the formulæ shown in this paper.

far as they contained no other reducible substance, at least none which would cause an overlapping of the titration curve or show a confusing color effect. As the course of the titration curves is strictly that of simple reversible systems we may also exclude the possibility that any of these dyes might be a mixture of different chemical individuals with overlapping curves. These three dyes are the following.

1. Gallocyanine[2] (Köchlin, 1881).



This is an oxazine and is of amphoteric nature. It is easily soluble at alkaline and at strongly acid reaction, but very slightly soluble between pH 5.5 and 3.5. The solutions have a red-violet color at pH < 4, blue from pH 5.5 to about 8, and red-violet at pH > 8. The insoluble form is obviously that of the isoelectric compound. The red form at strongly acid reaction is the positive ion, ionized at the dimethylamino group, the blue state is the monovalent ion due to the carboxyl group, and the red form at pH > 8 is a bivalent anion due to the hydroxyl group. The reduced form contains an additional hydroxyl group, and, being a polyphenol, it reacts with boric acid to give a complex acid. This prevents the use of the borate buffer in calibrating the constants of the dye. That range of pH (around 8) which is covered only by the borate buffer happens to be a very important range for the characterization of the dye, as can be seen from the curve in Fig. 3. In order to fill the gap due to this deficiency of the borate buffer we used successfully the veronal buffer recently described (6).

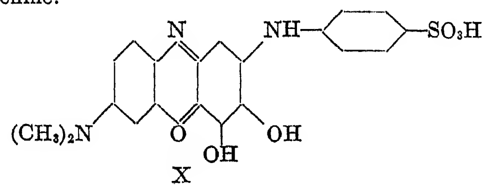
From the properties mentioned above it is easily understood that gallocyanine is very slightly soluble in pure water. The dye can be easily dissolved, however, in any buffer outside the range of pH 4 to 5. For the measurements presently to be described the dye was directly dissolved in the buffer used for the particular experiment.

The potential range of gallocyanine is rather close to that of

[2] See Schultz (4) No. 626, or (5) No. 883.

methylene blue, a desirable property in view of the known inadequacies of the latter and the importance of the range it covers (1, 7). Moreover, such a duplication is desirable because of the fact that methylene blue, and the similar Lauth's violet and toluylene blue, are basic dyes, whereas gallocyanine at the pH range of biological importance acts as an acid dye. This dye may therefore be very useful in biology.

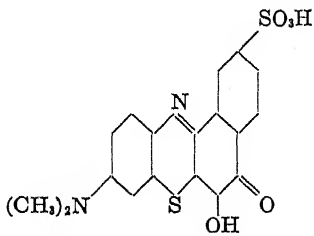
2. Gallophenine.[3]



This is a sulfonated oxazine. X stands for a monovalent acid residue.[4] It is easily soluble at any pH. It is usually blue, but turns green at very strong acid reaction and purple in a strong alkaline solution.[5]

The potential range of this dye is somewhat more negative than that of indigo disulfonate, approximating closely that of indigo monosulfonate. It is by far superior to indigo monosulfonate because of its high solubility. The solubility of indigo monosulfonate is so slight that Sullivan, Cohen, and Clark had to use approximate data concerning potentials. For this reason gallophenine may serve as a very desirable substitute for indigo monosulfonate.

3. Brilliant alizarin blue[6] (B. Heymann).



[3] *Cf.* (5) No. 879.

[4] This formula has been written here as an *o*-quinoid structure. One could write it just as well in the form of a *p*-quinoid structure.

[5] This dye, in Schultz' Farbstofftabellen, is mentioned only in the index, not in the text.

[6] See Schultz (4) No. 667, or (5) No. 931.

This is a sulfonated thiazine. Its solubility in pure water is, though not high, entirely sufficient. When the dye is added to pure cold water, there is an undissolved residue; but when the filtrate is mixed with a buffer of pH around 7 it remains clear and behaves as a true homogeneous solution. Especially the titration curve, as described in the following, precisely fits the course expected for a truly dissolved and completely reversible dye, if the concentration of the dye is small enough. The filtered pure aqueous solution is the adequate form of a stock solution and should be mixed with the buffer solution desired immediately before use.

The dye has a potential range more negative than any of the indigo sulfonates and therefore represents a valuable extension of the series of really reversible redox indicators, though there may be cases where the peculiarity of this dye with regard to its solubility may cause difficulties.

The solution of the dye is blue-violet in water. At very low pH (approximately 1) it changes a little towards blue. The violet becomes more pronouncedly blue at pH 7, and remains so down to very alkaline ranges.

Methods Used for Titrations.

The methods of tritration have been so carefully described by Clark that only little need be said in this respect. We wish, however, to add two items, one of perhaps only theoretical, the other of practical significance.

1. Use of Mercury Electrode in Presence of Hydrogen Gas.—When the oxidized form of the dye is reduced by a reductant such as sodium hydrosulfite, in many cases the mercury electrode can be used as an indifferent electrode even in an atmosphere of hydrogen gas. Mercury shows no inclination to work as a hydrogen electrode in the presence of hydrogen gas, and the potential of the reversible redox system can therefore be measured at the mercury electrode provided this potential is negative enough to reduce or perhaps eliminate by complex formation those traces of mercury ions which may be formed in the beginning of the experiment, and, on the other hand, is not too negative, the limit in this respect being set by the fact that even pure hydrosulfite shifts the potential of the mercury electrode no further than a poorly defined value 100

millivolts or so more positive than the hydrogen electrode at the same pH. The use of the mercury electrode is therefore re-

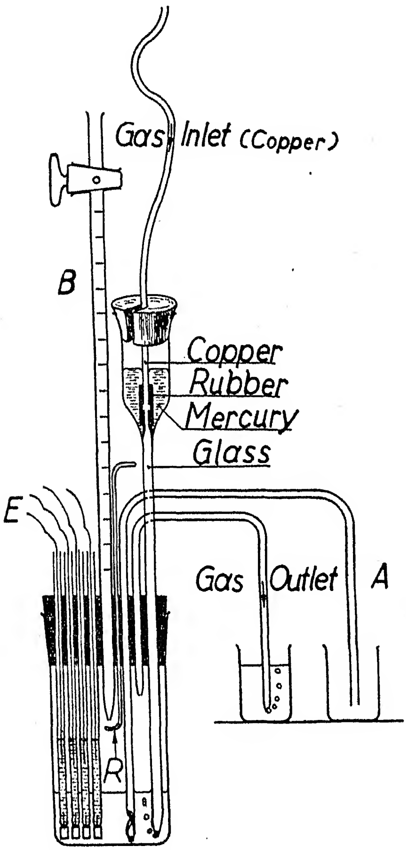


FIG. 1. Schematic drawing of the electrode vessel. E, electrodes; B, burette (notice the glass rod, R, next to the burette. It serves to catch the drop of fluid adhering to the tip of the pipette); A, agar lever, saturated with KCl (notice the stop-cock at its lower end). The whole vessel is held by a clamp which, by means of a motor, can bring about circular movements of the whole vessel around a perpendicular axis which lies outside the central axis of the vessel. The gas inlet is a flexible copper tubing allowing the electrode vessel to be moved freely.

stricted to a potential range, at pH 7, from about +0.1 to −0.2 or −0.25 volt, referred to the normal H_2 electrode. A great many

of the preliminary measurements for orientation were made by this method.

2. Use of Colloidal Palladium for Titration.—Most of the measurements were performed in the following way. The electrode vessel (Fig. 1) was a cylindrical bottle, of such dimensions that the solution to be titrated (25–30 cc.) occupied a depth of only about 3 to 4 cm., to allow stirring. The bottle was closed by a rubber stopper through which were fixed:

A glass tube as a gas inlet. The gases were purified over heated copper, as described by Michaelis and Flexner (8), and conducted through copper tubing. As the description of the connection of the gas inlet in the paper just mentioned seems a little too brief, it may be given in more detail here. The copper tubing coming from the furnace is fixed as shown in Fig. 1. The opening is mounted with a short rubber tubing (Fig. 1). This is stripped over glass tubing and the glass calix is filled with mercury. A loosely fitting rubber stopper with a slit holds the copper tubing in place and prevents the mercury from splashing when the whole apparatus is being stirred.

A glass tube as gas outlet.

An agar tube saturated with KCl as bridge. The most suitable form, which at the same time leaves a minimum of contact surface for diffusion, was this. The glass lever (A, Fig. 1) is open at the end but carries a not too well ground stopper at the other end. The handle of the stopper should not have a larger diameter than the glass tube, so that it can be pushed through the hole of the rubber stopper of the electrode vessel. This glass stopper has on its upper end a hook and the glass tube contains inside a small thickened spot which serves as a support for the hook. The stopper is turned into the fitting position. The whole lever is filled with agar saturated with KCl, and the glass stopper fitted in, not too tightly, so as to leave a capillary agar connection. This stopper, after use, may be regenerated by keeping it under saturated KCl solution, without its being necessary to refill the whole tube each time.

A platinized platinum electrode, by which the pH of the solution after complete reduction is measured directly. On measuring the pH directly one need not rely on the pH as calculated from the composition of the buffer. One may work then even with buffers

which are not standardized; *e. g.*, with phosphates of a concentration other than that of the standard solution. This is especially important when a dye has to be dissolved in pure water and a buffer is added later.

Three blank electrodes, say two of blank platinum and a gold-plated one.

A burette (B) calibrated in hundredths of a cc., the glass cock being located close to the upper end, above the zero point of the graduation.

It is very useful to have a glass rod (R) with small spoon-like hook. This rod should fit tightly, but be movable. It serves to catch an adhering drop of the oxidant from the tip of the burette during the titration.

In the beginning of the experiment 0.1 to 0.3 cc. of a solution of colloidal palladium according to Nacht-Pahl (1 gm. to a liter of water) is added and the reduction is carried out by means of a stream of hydrogen. After complete reduction the pH is measured with the platinized electrode. Now the gas lead is shifted to nitrogen, and the hydrogen is expelled from the solution and from the palladium. The potential of the platinized electrode is watched during this period. The removal of the hydrogen gas may safely be considered as complete for any practical purpose when the potential of the platinized electrode has become more positive by 80 to 100 millivolts. The crucial test for the complete removal of hydrogen is the observation that the very first drop of the oxidant added to the solution brings about not only an immediate coloration, but a coloration which persists indefinitely. A trace of hydrogen that may have remained will reduce the dye over again.

One may be surprized that the removal of hydrogen from palladium can be brought about so completely in a relatively short period of time (30 to 60 minutes). One may, however, take into consideration the fact that the total amount of palladium contained in the solution is a small fraction of 1 mg. and that the ultramicroscopic particles of palladium are brought to absorption equilibrium with the surrounding gas much more quickly than a macroscopic particle of the metal.

As this electrode vessel is attached to the gas inlet in an elastic, flexible manner, the stirring can be produced by shaking, and there is no need for a stirring rod with all its inconveniences. The

stirring, after each addition of the content of the burette, may be done by hand, or better, by a shaking apparatus which executes circular movements of the vessel around a perpendicular axis. This arrangement allows one to read the potential either at rest or during the shaking and to study the influence of shaking. In well poised systems, there is a very small but distinct difference, noticeable even in the case of a hydrogen electrode with platinized platinum. Usually the potential is more negative by some tenths of a millivolt at rest than while being shaken. The potential at rest is to be considered as the desired value, because the thermodynamic theory on which the establishment of the potential is based does not take into account a mechanical movement of the electrode relative to the liquid in contact with it, which may interfere mechanically with the establishment of the electric double layer responsible for the potential difference. On the other hand without a thorough previous shaking there is no guarantee of a definite state of equilibrium throughout the whole system including the surface layer of the liquid immediately adjacent to the electrode.

The whole apparatus, consisting of the electrode vessel, a great part of the copper leads for the gases, the shaking apparatus, and the calomel electrodes, was kept in a compartment of constant temperature large enough to work in conveniently. The temperature was controlled by a toluene-mercury regulator, the heat being furnished from freely suspended heating wires of nichrome, the heat of which was blown off by an electric fan. The temperature of a bottle with water of the heat capacity of the electrode vessel was constant within much less than 0.1°, the constant temperatures being reached within 20 to 30 minutes. Almost all these experiments were made at 25°. Some experiments at 35° show that the temperature coefficients of the dye potentials are very small.

In most cases quinone was used as oxidant, sometimes also phenol indophenol or ferricyanide. The solution should be deaerated before pouring it into the burette. The air may be removed simply from the quinone solution by briefly boiling and immediately sucking it into the burette.

Result of Titration Experiments.

1. Gallocyanine.—Fig. 2 shows one example of a titration experiment at constant pH. The dye is dissolved in a phosphate buffer,

and quinone is used as oxidant. The dye is reduced by H_2 and palladium as described before, pH is measured with the platinized electrode, H_2 is expelled by purified N_2, and the titration is performed. Three electrodes, two of blank platinum and one of gold-plated platinum agreed, with respect to the potential, within a few tenths of a millivolt, except for the very beginning and the very end of the titration where the disagreement between different

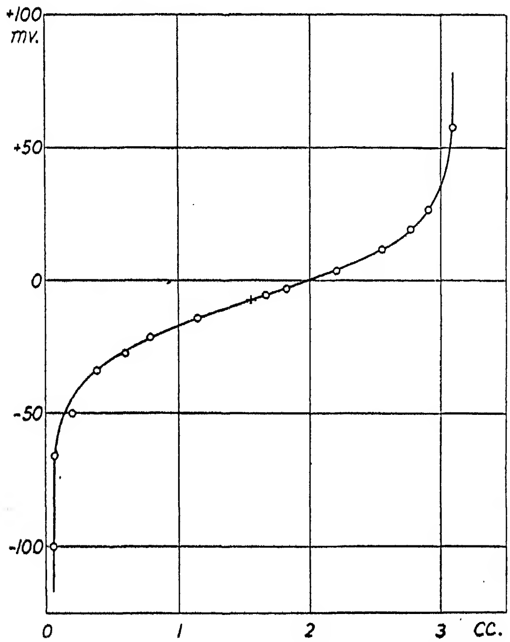


Fig. 2. Gallocyanine, reduced and then titrated with quinone, at 25.0°. pH = 7.390, phosphate buffer; abscissas denote cc. of quinone solution, ordinates, potential referred to the normal hydrogen electrode, in millivolts.

electrodes may be greater, but irrelevant for the shape of curve. The potential was established practically instantaneously after stirring, and was constant for any length of time. The difference between the potential reading during the stirring or in the state of rest was never sufficiently large to appear in the plotting on the scale in Fig. 2. The values at rest were used for the diagram. The drawn out curve is the one theoretically calculated according

to the principles set forth by Clark. From this curve E_0', or the potential of the dye in the half reduced state referred to the normal hydrogen electrode, is best evaluated graphically. The assumption underlying the calculation of pH is that the pH of standard acetate is 4.62. From this assumption the potential of the calomel electrode used referred to the normal hydrogen electrode, was calculated, and this standardization of the calomel electrode was frequently repeated during the course of the series of individual

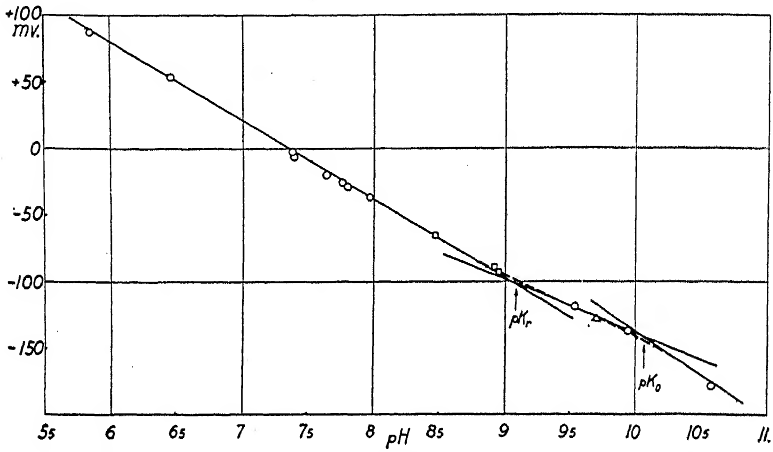


FIG. 3. Potential of gallocyanine in the half reduced state at varied pH, at 25.0°.

The abscissas denote pH; ordinates, potential, referred to the normal hydrogen electrode, in millivolts. Buffers, ○ phosphate, □ veronal, △ glycocoll. The curve as interpolated from the experiments is dotted. The tangents of 0.06 slope respectively 0.03 slope are drawn out. pK$_r$, pK$_o$, dissociation constants of the reduced form and the oxidized form respectively.

titration experiments. Only one titration experiment is shown in detail, but all the others were used similarly to calculate the E_0' at various pH graphically shown in Fig. 3. Borate buffer could not be used because the reduced form of the dye has two phenolic hydroxyl groups. Experiments with borate as buffer gave a curve deviating markedly from that expected for an undisturbed system of reversible dye, the curve being much too flat in the beginning. When, by fitting best values to such an unsatisfactory curve, a trial calculation of E_0' was made, a value quite outside that of the theo-

retical curve was found. None of the following buffers showed this specific effect: Sörensen's phosphate buffers, mixtures of secondary phosphate and NaOH, acetate buffer, veronal buffer (recently recommended (6)), and glycocoll buffer.

The diagram, Fig. 3, shows that the slope of 0.0591 volt shift per unit of pH holds between pH about 6 to 9. There is a deviation from this slope towards the acid side from 5.5. This part of the curve could not be investigated in more detail because at pH < 5.5

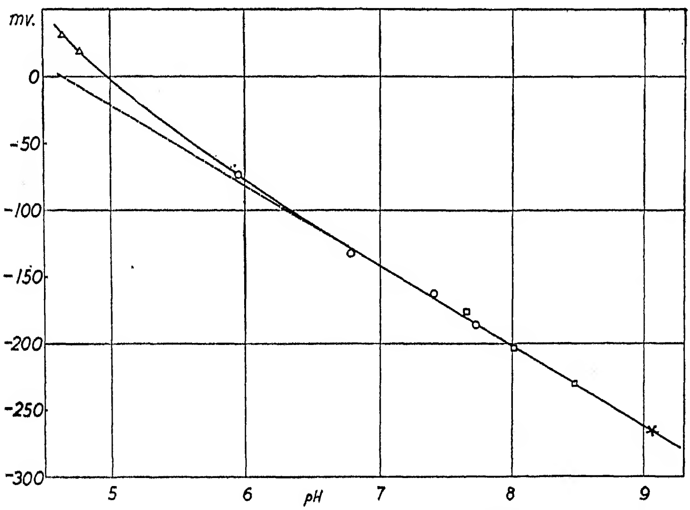


FIG. 4. Potential of gallophenine in the half reduced state, at varied pH, and at 25.0°. The abscissas denote pH; ordinates, potentials referred to the normal hydrogen electrode, in millivolts. Buffers, △ acetate, ○ phosphate, □ veronal, * glycocoll.

we approach that range of pH in which the solubility of the dyes becomes too slight to allow accurate titration experiments, and the figures obtained in this range are omitted in the diagram. A second deviation is encountered between pH 9 and 10. This range of deviation of the slope coincides with the range of color change of dye from blue to red-violet, indicating that it is due to the effect of an acid or basic dissociation constant of the dye, as Clark has shown in many instances. On applying his principles one may calculate graphically a dissociation constant for the oxidized form

of the dye, pK_o, and another for the reduced form, pK_r. This is performed by drawing the best fitting tangent with the slope of $\frac{0.0591}{2}$ volt per unit of pH. The two points of intersection of such

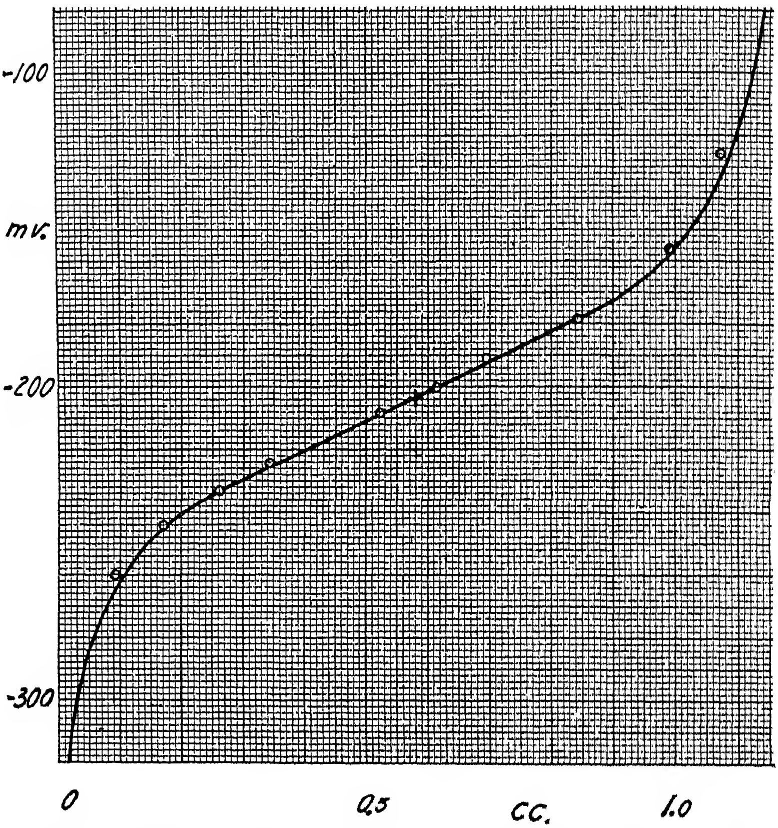


FIG. 5. Titration of reduced brilliant alizarin blue with quinone. pH = 5.918. Abscissas denote cc. of quinone solution, ordinates, potential in millivolts referred to the normal hydrogen electrode.

tangents give the two dissociation constants at the abscissa. The first of these, $pK_r = 9.1$ owing to the fact that the curve is flattened at this region, according to the theory belongs to the reduced form, the second, $pK_o = 10.1$, to the oxidized form, because the curve becomes steeper.

2. Gallophenine.—The data for E_0' at various pH are plotted in Fig. 4. The curve has a slope of 0.0591 volt per pH unit, and the observed points fit into this straight line throughout the whole range of pH investigated, except for pH <6, where a dissociation constant of the dye obviously interferes.

3. Brilliant Alizarin Blue G.—Here one individual titration experiment is shown in detail (Fig. 5), and the result of all experi-

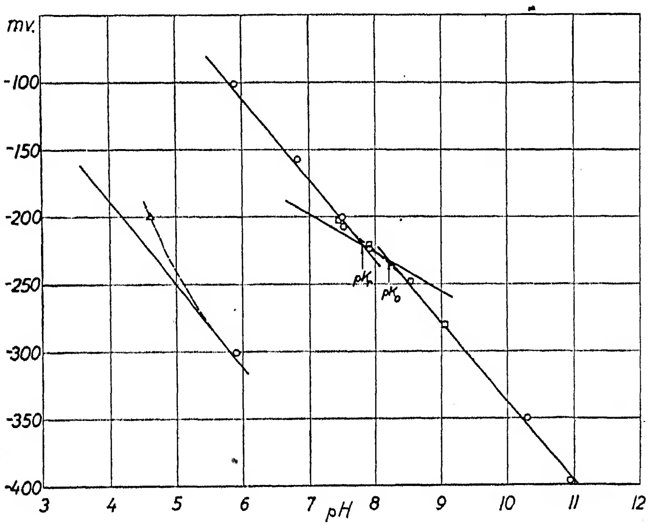


FIG. 6. Potential of brilliant alizarin blue, in the half reduced state at varied pH, at 25.0°. The abscissas denote pH, ordinates, potential in millivolts, referred to the normal hydrogen electrode. The left-hand curve is an extension of the right-hand curve to the left, with respectively shifted ordinates, to save space. The drawn out lines are the tangents with the slope 0.0591 respectively 0.02905. pK_r, pK_o are dissociation constants of the reduced form and the oxidized form respectively. Buffers, ○ phosphate, □ veronal, △ acetate.

ments is plotted in Fig. 6. The slope is regular between at least pH 5.5 and 7.8 within the limits of error, then flattens and regains the normal slope from about 8.6 into the most alkaline range investigated. The intermediary part of the curve, around pH 9, could be studied only by means of the veronal buffer. The points around pH 7.5, where the phosphate and the veronal buffers overlap, were studied with either buffer, and a perfect agreement was obtained.

On applying the graphic construction mentioned above one obtains as a dissociation constant for the reduced dye $pK_r = 7.8$, and for the oxidized dye $pK_o = 8.2$. The deviation from the straight line throughout the pH range 6 to 11 is small indeed. The straight line between pH 6 and 8, and the one between 9 and 11, are only 0.1 unit of pH apart. Yet we do not believe this separation to

TABLE I.

Potentials of the Half Reduced Dyes Referred to the Normal Hydrogen Electrode at 25°.

pH	Brilliant alizarin blue.	Gallophenine.	Indigo disulfonate.	Methylene blue.	Gallocyanine.
5	−0.040	−0.003	−0.10	+0.101	
5.25	−0.062	−0.023			
5.5	−0.080	−0.042			+0.110
5.75	−0.097	−0.060			+0.095
6	−0.112	−0.077	−0.069	+0.047	+0.080
6.25	−0.127	−0.094			+0.065
6.5	−0.143	−0.110			+0.051
6.75	−0.157	−0.127			+0.46
7	−0.173	−0.142	−0.125	+0.011	+0.021
7.25	−0.188	−0.157			+0.007
7.5	−0.203	−0.172			−0.008
7.75	−0.216	−0.187			−0.023
8	−0.226	−0.202	−0.167	−0.020	−0.037
8.25	−0.237	−0.217			−0.042
8.5	−0.250	−0.232			−0.067
8.75	−0.265	−0.247			−0.082
9	−0.279	−0.262	−0.199	−0.050	−0.095
9.25	−0.293	−0.276			−0.107
9.50	−0.309	−0.291			−0.118
9.75	−0.323				−0.128
10	−0.337				−0.140

The values for indigo disulfonate and methylene blue are shown for comparison and have been taken from the measurements of Clark and his associates, at 30°.

be within the errors of the experiments; even when we allow for errors within reasonable limits it is impossible to connect all observed points from pH 6 to 11 by one straight line of the slope 0.0591 per unit of pH.

In using these dyes as redox indicators the following suggestions

with regard to solubility may be made. Gallocyanine is best dissolved directly in the buffer to be used. Gallophenine is so easily soluble under any condition that no special precautions are necessary. Brilliant alizarin blue is best dissolved in pure water, filtered, and this filtrate may be used as a stock solution and added to the buffer to be used. If prepared in this way the solution of the dye in the buffer behaves as a true homogeneous, non-colloidal solution, as can be inferred from the titration curves.

All these experiments were made at 25° ± 0.05°.

A few experiments were made at 35°, and the temperature coefficient turned out to be very small. Not enough data are available as yet to give accurate figures. Table I gives the E_0'; *i.e.*, the potential of the half reduced dye, at 25° and at various pH, and some comparative data at 30° for the dyes of Clark's series.

SUMMARY.

Three new redox indicators are characterized, and their potentials summarized in Table I.

Technical details for general measurement of reversible redox systems and their titration are described.

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THE WILLIAMS-WATERMAN VITAMIN B_3.

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In a previous communication, Williams and Waterman (1) submitted evidence that polyneuritic pigeons, made so by either an exclusive diet of polished rice or a synthetic diet complete in all factors except the vitamin B complex, require for full weight restoration or maintenance a factor that is distinct in properties and distribution from the antineuritic factor (vitamin B_1 or B), or the antipellagric factor (vitamin B_2, G, or P-P). This factor they consider more heat-labile than either of these vitamin B fractions. To it they assigned the provisional designation vitamin B_3.

The factor is not to be confused with the recently described vitamin B_3 of Reader (2). Of this factor Miss Reader says: "Moreover, certain preliminary experiments carried out in this department (Peters, private communication), indicate that the second rat factor does not restore the weight of pigeons; thus the evidence seems to be in favour of two further factors—one, reported by Williams and Waterman, for pigeons: the other, reported in this paper, for rats."

In his recently published (3) Harben Lectures Peters has not only supported the Williams-Waterman claim but advanced evidence to support their contention.

The purpose of the present paper is to present further data bearing on the distribution and properties of the Williams-Waterman factor designated in what follows as vitamin B_3.

Method of Assay.

Tables I and II indicate both the distribution of vitamin B_3 in selected foodstuffs and the method of determining its presence and

concentration. Adult pigeons made polyneuritic with polished rice or by a synthetic diet complete in all known factors except vitamin B complex, are promptly cured of polyneuritis and restored to normal weight by the addition of whole grains or yeast to the diet. Both of these effects were originally considered essential for demonstration of the presence of vitamin B. If, however, such pigeons are supplied with vitamin B_1 factor only (either in the form of Williams and Waterman Y concentrate (1), Kinnersley and Peters' antineuritic concentrate (4), or Jansen and Donath's crystalline B (5)[1]), polyneuritis is promptly cured but weight restoration fails. Increasing the vitamin B_1 factor does not effect such weight restoration (see foodstuff Controls A and B, Table I). Such increase over the amount necessary to remedy polyneuritis has been tested at as high as 20 times the curative dose without appreciably affecting weight restoration.

The results obtained in Tables I and II were secured by placing pigeons on polished rice until well advanced toward polyneuritis, then by adding an amount of vitamin B_1 adequate to prevent the polyneuritis. After 10 to 14 days on the B_1 supplement alone, measured amounts of the food to be assayed were added and their effect on weight restoration noted. The extent of weight increase obviously indicated relative richness of the foodstuff in B_3 or whatever is the weight-restoring factor. In some cases the B_1 supplement was omitted at a later stage to determine whether the foodstuff possessed adequate B_1 itself. The results in Table I are self-explanatory, but they indicate among other things that certain fairly good sources of B_1 are not equally rich in B_3 and *vice versa*. Milk, fruit juices, vegetable juices, and cane molasses were all inferior to beef liver and lean beef in this B_3 factor. Temperatures as low as 60° appreciably decrease the B_3 potency of good sources such as barley and yeast. It is also evident that weight restoration, while requiring B_3, is not possible in the absence of adequate B_1 regardless of the richness of the source in B_3.

Heat and Alkali Sensitivity of Vitamin B_3.

Chart I is offered as evidence that yeast can be reduced in vitamin B_3 content by a preliminary treatment with alkali without

[1] The fact that the Jansen-Donath crystals are antineuritic and not weight-restorative has been confirmed in our laboratory with crystals kindly supplied to R. R. Williams by B. C. P. Jansen.

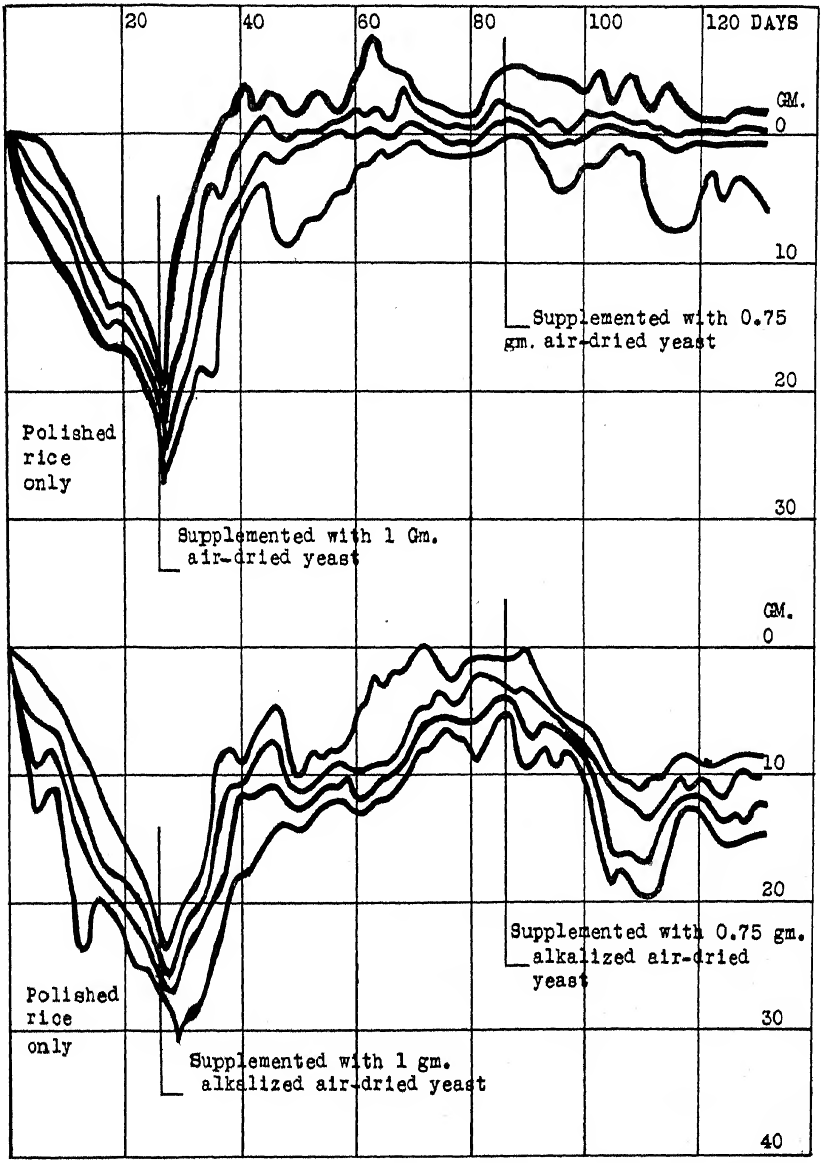

CHART I. Treating yeast with alkali before drying destroys vitamin B_3 even though the drying temperature is kept at 20°.

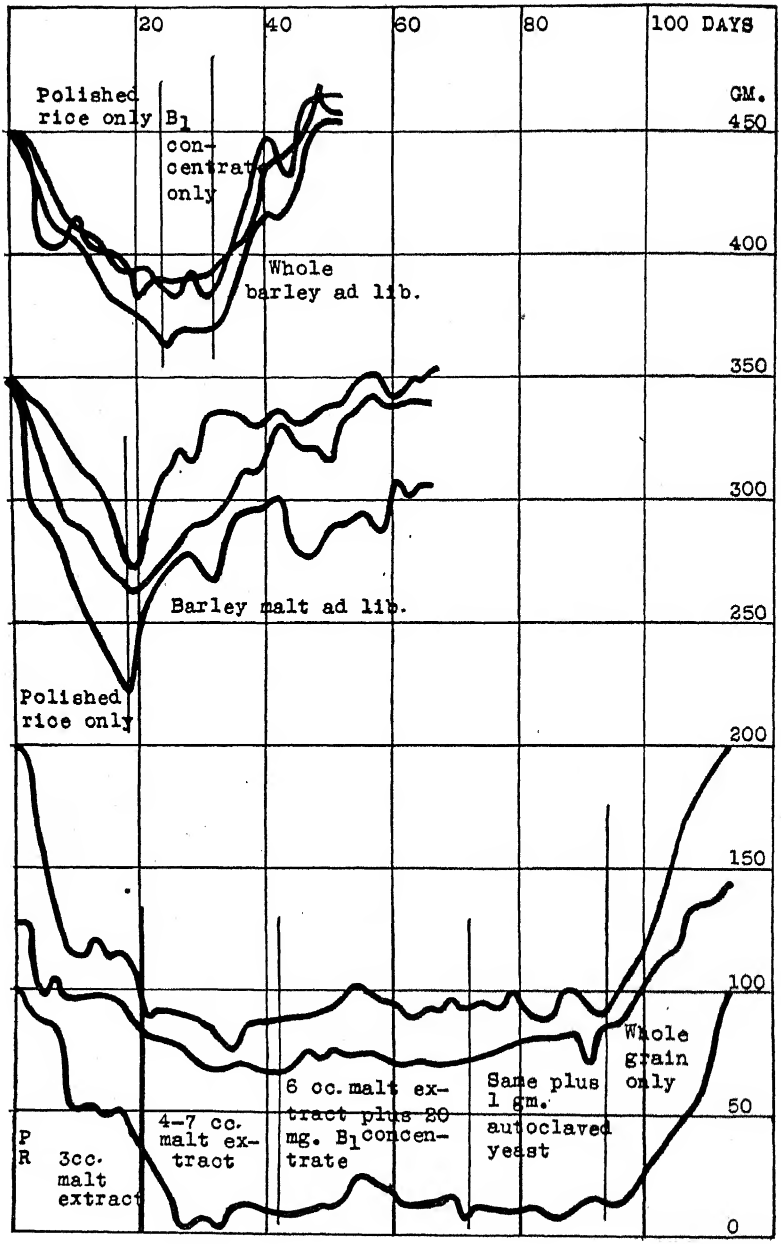

CHART II. The vitamin B_3 content of whole barley is lost through the process of malt extract manufacture.

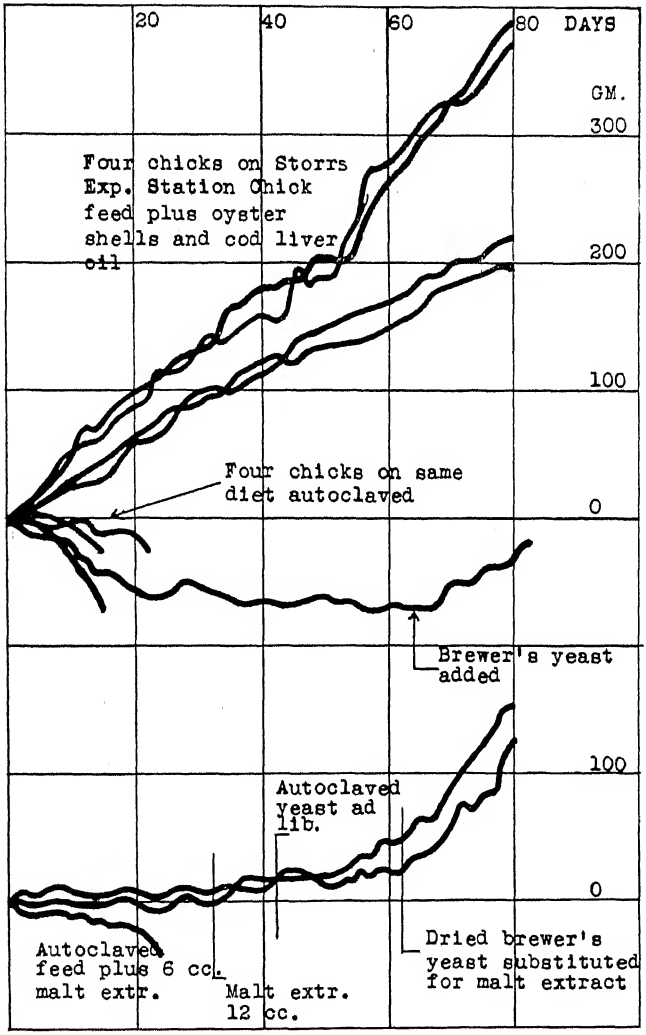

CHART III. Autoclaving chick feed destroys vitamin B_3. See foot-note 2 for composition of the Storrs chick feed.

change in drying temperature and without reducing antineuritic potency below that necessary to protection. Chart II contrasts the B_3 potency of barley, offered as whole grain in malted form, and

as malt extract, with progressive decrease in B_3 value. Temperatures were controlled in this series of preparations and the malt extract did not go above 60° in manufacture. It is evident from the charted results that this temperature had little effect on B_1 content, but was almost completely destructive of B_3. This series also shows that the weight restoration attributed to B_3 is not a property of B_2 (G), for autoclaved yeast had no effect on the weight restoration.

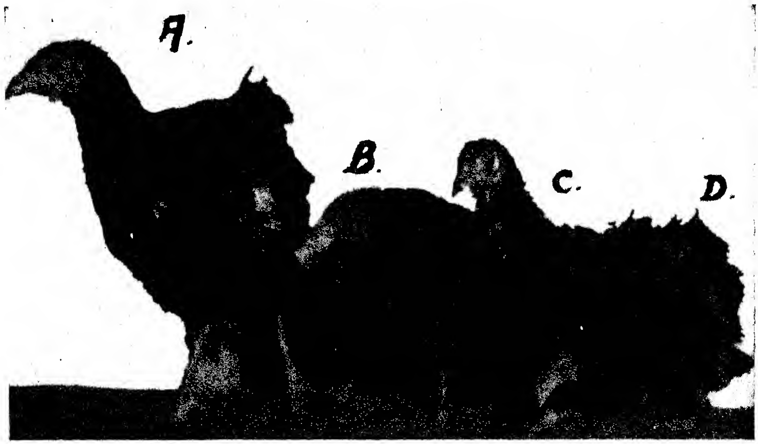

FIG. 1. Contrast in growth of chicks when supplied with both vitamins B_1 and B_3 and when deprived of vitamin B_3 but adequately supplied with B_1. Chick A was on the Storrs' chick feed plus cod liver oil and ground oyster shells. The ration contains both vitamins B_1 and B_3. Chick B was given an autoclaved supplement with vitamin B_1 but without B_3. Chick C was on autoclaved feed supplemented with malt extract. Chick D was given autoclaved feed with no supplements.

Vitamin B_3 is a Bird Requirement.

In none of our tests have we been able to show the need of vitamin B_3 on the part of rats. These animals supplied with B_1 concentrate and autoclaved yeast (B_2 or G), alone as vitamin B supplements to a basal ration complete in all other known factors provided by the usual casein-containing Osborne and Mendel or Sherman diets, attain normal growth curves. Our indications of a B_3 deficiency were first obtained from the pigeon studies. This

led us to study another type of bird and for that purpose we selected the growing chick. Charts III and IV and Fig. 1 summarize findings that seem to demonstrate the need for B_3 on the part of these birds.

Chart III summarizes findings with Rhode Island Red chicks fed on the chick feed mixture suggested by Storrs Agricultural

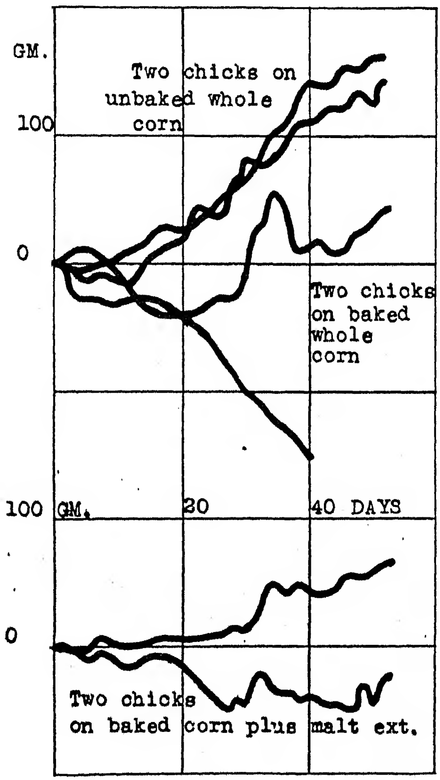

CHART IV. Baking corn destroys vitamin B_3.

Experiment Station[2] supplemented with cod liver oil and ground oyster shells. Four chicks were placed on this mixture. While they did not attain full normal growth in our laboratory test, their growth response when contrasted with four other chicks on the

[2] This is composed of fine cracked corn, 150 pounds; fine cracked wheat, 150 pounds; cut oatmeal, 100 pounds.

same diet autoclaved before feeding, showed that this heat treatment destroyed factors important to the chick. That the factor destroyed is not solely the antineuritic vitamin B_1 and the antipellagric vitamin B_2 or G became evident when the autoclaved feed supplemented by B_1 and B_2 failed to restore growth though protecting fully from polyneuritis. Fig. 1 shows that the difference due to destruction of some factor other than B_1 or B_2 (G) is very significant.

Chart IV shows similar results with chicks fed whole corn and baked whole corn supplemented with vitamin B_1 adequate for protection from polyneuritis in the form of malt extract.

Unlike the adult pigeons, the growing chicks showed some need for vitamin B_2 (G) as a slight weight increase followed the supplement with autoclaved yeast.

SUMMARY.

New evidence is submitted to demonstrate further the existence of a bird growth and weight restoration factor provisionally designated by Williams and Waterman as vitamin B_3, and distinct in distribution and properties from the antineuritic factor (B_1 or B) or the antipellagric factor (B_2 or G).

Yeast, whole grains, and malt are good sources of vitamin B_3, but while malt extract often retains a good concentration of B_1, its manufacture practically eliminates B_3. Beef and beef liver are fair sources of B_3 and distinctly superior in this factor to milk, orange and tomato juice, spinach, and potato juice or cane molasses.

Chicks require this factor for growth, even when fully supplied with B_1 and B_2 factors and with other nutrients and vitamins.

Vitamin B_3 is much more heat-labile than B_1 and if submitted to alkali treatment before drying, temperatures as low as 20° will markedly reduce the yeast content of this factor. Malt extracts made at temperatures as low as 60° are practically devoid of B_3 though still very effective as sources of B_1.

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CHEMICAL STUDIES ON TOAD POISONS.

II. CH'AN SU, THE DRIED VENOM OF THE CHINESE TOAD.*

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INTRODUCTION.

In a previous communication (1), in which we reported the isolation of epinephrine from Ch'an Su, we have already mentioned that our reasons for taking up this investigation were the following: first, the chemical results so far obtained on Ch'an Su, which has been investigated under the name of Senso by several Japanese chemists (2, 3), differ widely, and, secondly, we wished to see if Ch'an Su also contains a substance similar in its chemical composition to the bufotoxin isolated by Wieland and Alles (4) from the European toad, *Bufo vulgaris*. In a preliminary publication (5) we have already reported that we found good evidence of the presence of such a compound in Ch'an Su. We shall describe in this paper the isolation of this nitrogen-containing body which we call cinobufotoxin. At the same time we also prepared from Ch'an Su "bufagin" which was mainly the substance isolated and worked on by the Japanese chemists (2, 3) who so far have failed to show the presence in Ch'an Su of a nitrogen-containing body similar to bufotoxin. We found that the "bufagin" from Ch'an Su is different from the bufagin isolated by Abel and Macht (6) from the tropical toad, *Bufo agua* (*Bufo marinus*). We shall therefore for the sake of differentiation call the "bufagin" from Ch'an Su cinobufagin. The analytical data obtained for cinobufagin agree with those reported by Kotake (3), and we shall therefore give for the present the same empirical formula $C_{29}H_{38}O_7$

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as Kotake gives for cinobufagin. We would like, however, to say here that the empirical formulas presented in this paper for cinobufotoxin, cinobufagin, and derivatives are given with reservations, as it is quite possible that they may have to be revised as the chemical work on these compounds continues.

Contrary to Kotake (3) we find that only one acetyl group is introduced into the molecule of cinobufagin when this is acetylated. Oxidation of cinobufagin with chromic acid yields a mono-ketone, thus indicating the presence of a secondary hydroxyl group. When cinobufagin is treated with an alcoholic solution of potassium hydroxide an acid results which could not be obtained in crystalline form. The formation of this acid occurs with the opening of a lactone ring and splitting off of acetic acid, giving rise to a hydroxycarboxylic acid. The analytical data obtained agree fairly well with the formula $C_{27}H_{38}O_7$, the following reaction taking place,

$$C_{29}H_{38}O_7 \xrightarrow{+\ 2H_2O} C_{27}O_{38}H_7 + CH_3COOH.$$

Under the influence of concentrated hydrochloric acid on cinobufagin acetic acid and water are split off. A yellow substance is formed which unfortunately could not be crystallized and which seems to contain chlorine. On boiling the latter compound with acetic anhydride and anhydrous sodium acetate, a chlorine-free substance results which can be obtained in crystalline form. The elucidation of the chemical composition of this body requires further work. From the foregoing one can conclude that the molecule of cinobufagin contains a lactone, an acetoxyl, a secondary hydroxyl, and at least one tertiary hydroxyl group. As to the nature of the 7th oxygen atom in the molecule of cinobufagin nothing can be said with certainty. Most of this observed behavior of cinobufagin is in agreement with the findings of Kotake (3).

The empirical formula of cinobufotoxin is represented by $C_{43}H_{64}O_{12}N_4$. We have to admit that we derived this formula by simply adding the empirical formula of 1 molecule of suberyl-arginine to the empirical formula of 1 molecule of cinobufagin, since cinobufotoxin is made up of 1 molecule of cinobufagin and 1 molecule of suberyl-arginine, as will be shown later in this paper. The analytical data obtained do not agree with this formula.

It seems quite difficult to obtain an absolutely colorless preparation of cinobufotoxin. As soon as we have more material we hope to be able to settle this point. Hydrolysis of cinobufotoxin with 50 per cent hydrochloric acid yielded a decomposition product of cinobufagin which so far we have not been able to isolate in crystalline form (see treatment of cinobufagin with concentrated hydrochloric acid), arginine, which was obtained as a flavianate, and, furthermore, suberic acid, which was identified as such. The isolation of arginine and suberic acid from the hydrolytic split-products of cinobufotoxin indicates a close chemical relationship of cinobufotoxin to the bufotoxin of Wieland and Alles (4).

The reaction may be represented as taking place in the following steps:

Cinobufotoxin → cinobufagin + suberyl-arginine.
Cinobufagin → cinobufagien[1] + acetic acid + water.
Suberyl-arginine → arginine + suberic acid.

The pharmacological study of cinobufotoxin and cinobufagin revealed that these two compounds show a digitalis-like action. Cinobufotoxin was found to be more toxic than cinobufagin.

Suberic acid was also isolated from one fraction of material obtained by working up Ch'an Su. It is a question whether this acid exists as such in the dried secretion or whether it is formed by the decomposition of cinobufotoxin.

By passing hydrochloric acid gas into the mother liquor obtained in the preparation of cinobufagin a chlorine-containing substance can be prepared which was thought by Kotake (3) to be a derivative of cinobufagin. We have accumulated evidence, however, which makes us believe that this chlorine compound is not derived from cinobufagin but from bufotalin, which has been obtained by Wieland and coworkers (4, 7) also from the European toad, *Bufo vulgaris*. We found that by passing hydrochloric acid gas into an alcohol-ether solution of pure cinobufagin a quite different compound than that obtained from the mother liquor is formed. Unfortunately, we were not able to crystallize this body, which also contains chlorine. By treating it with acetic anhydride

[1] We call the product obtained by treating cinobufagin with hydrochloric acid cinobufagien in conformity with the nomenclature employed by Wieland for the product obtained from bufotalin under the same conditions.

and anhydrous sodium acetate one can obtain a crystalline substance which is free of chlorine. This compound awaits further investigation. We have so far not succeeded in converting the chlorine-containing substance obtained from the mother liquors of the cinobufagin preparation into a known derivative of bufotalin in order to get a direct comparison. The analytical data obtained for this substance agree well with a product derived from bufotalin by splitting out acetyl and replacing a hydroxyl group by chlorine.

$$C_{26}H_{36}O_6 \xrightarrow[H_2O]{HCl} C_{24}H_{33}O_4Cl + CH_3COOH$$

Furthermore Kotake (3) also prepared in the same manner from the secretion of the Japanese toad a chlorine-containing substance which seems to be quite similar to the compound obtained from Ch'an Su. Later Kotake (8) was able to show that the secretion of the Japanese toad contains bufotalin. We believe that the two chlorine-containing substances prepared by Kotake from Ch'an Su and the Japanese toad are the same and derived from bufotalin. Further work is required to settle this point beyond doubt. At the present we are engaged in seeing if we can isolate bufotalin from Ch'an Su. By treating this with hydrochloric acid gas one should obtain a chlorine-containing substance identical with that prepared from the mother liquors of the cinobufagin preparation.

In addition, we were recently successful in isolating from one fraction of the material obtained in working up Ch'an Su a crystalline body, in the form of a flavianate, which seems to be identical with the pressor principle isolated by Handovsky (9) from the secretion of the species of toad which was investigated by Wieland and coworkers. We believe, however, that the salts described by Handovsky are not derived from the body which is originally present in the secretion but are formed from the decomposition products resulting from the original substance by the action of alkali. As soon as we have more of this substance at our disposal we shall try to elucidate its chemical composition. The pharmacological actions of our compound agree well qualitatively with those reported by Handovsky, but it seems that our product is much more active than that of Handovsky. The finding in Ch'an Su of this

pressor principle which seems to be identical with that in the European toad further strengthens our belief that Ch'an Su may also contain bufotalin.

Further work is in progress on the oxidative degradation of cinobufagin as well as on attempts to find a chemical correlation with the digitalis glucosides, to which its pharmacological action is similar.

Most of the analyses reported in this paper were carried out by Dr. Ing. A. Schoeller, Berlin-Schmargendorf, Germany.

EXPERIMENTAL.

400 gm. of powdered Ch'an Su mixed with 1 liter of 96 per cent ethyl alcohol were allowed to stand at room temperature for 14 days. After filtration the residue was again extracted with 1 liter of 96 per cent ethyl alcohol at 50° for 10 hours. The two alcoholic filtrates were combined and evaporated down at room temperature under a fan to about 300 cc. of liquid and let stand overnight in the ice box. Crystalline material precipitated out and was filtered off. It was found that the crystals obtained were cholesterol. The filtrate from the cholesterol fraction was further evaporated *in vacuo* to about 100 cc. of liquid, and about 1 liter of distilled water was added with thorough stirring. A gummy mass precipitated out which on being mixed well with water gradually became brittle. After standing overnight, the precipitate was filtered with suction or centrifuged off and dried at room temperature, Preparation A.

The aqueous filtrate from Preparation A was evaporated *in vacuo* to about 200 cc. and then shaken out with chloroform to remove organic matter like cinobufagin and other substances which were kept in suspension. The aqueous solution was acidified with hydrochloric acid and then shaken out several times with ether. After the ether solution was dried with anhydrous sodium sulfate and filtered, the ether was evaporated and the semicrystalline residue was recrystallized from dilute ethyl alcohol. There were thus obtained colorless prism-shaped crystals which by their melting point (141°), mixed melting point with suberic acid, and by analysis could be identified as suberic acid.

For analysis the substance was dried in a desiccator over $CaCl_2$.

4.826 mg. substance: 3.46 mg. H_2O, 9.760 mg. CO_2.
4.847 " " : 3.50 " " 9.795 " "

$C_8H_{14}O_4$.	Calculated.	C 55.17,	H 8.04.
	Found.	" 55.16,	" 8.02.
	"	" 55.12,	" 8.08.

Besides suberic acid the ether solution contained other fatty acids.

The aqueous solution which had been shaken out with ether was evaporated to about 100 cc. and after filtration an excess of flavianic acid was added and the whole heated on a water bath until a clear solution resulted. It was let stand in the ice box for several days, when a crystalline precipitate gradually settled out. After filtration the residue was recrystallized from water in which the salt is soluble in the heat but quite insoluble in the cold. The flavianate comes out from this solvent in long fine needles which melt at 205° with decomposition. The chemical properties of this compound agree with those given by Handovsky (9) for his pressor principle isolated from the European toad, *Bufo vulgaris*. In the introduction we have already stated that there is also a pharmacological similarity. We shall refrain from reporting the analytical data obtained for this flavianate, until more information regarding its chemical composition has been accumulated. By decomposing the flavianate with a sodium carbonate solution and extracting the alkaline solution with ether for 12 hours, a picrate could be prepared from the ether solution which melted at 165°; Handovsky gives 168° as the melting point for his picrate. In conformity with Handovsky we found that the ether extracted only a small amount of material, as most of it stayed in the alkaline solution. Probably decomposition takes place under the influence of alkali.

Precipitate A, obtained by adding water to the alcoholic extract of Ch'an Su, was dissolved in about 200 cc. of chloroform. After filtration from insoluble matter which was found to be mainly fatty acids (suberic acid could be isolated and identified) 200 cc. of ether were added to the chloroform solution. A dark gummy precipitate was formed from which the solution was filtered after standing about 1 hour. The precipitate was discarded as no

crystalline material could be obtained from it. To the chloroform-ether solution about 100 cc. more ether were added and after standing for 4 hours at room temperature, the solution was poured off from the precipitate. This precipitate was worked up separately, 200 cc. more ether were added to the solution, and the whole put in the ice box and let stand for 1 week. Cinobufotoxin precipitated out gradually in semicrystalline form. After filtration, the residue (Preparation B) containing the cinobufotoxin was purified further by dissolving it in absolute ethyl alcohol at 50° and adding ether after filtration and cooling. The ether precipitates out the cinobufotoxin, which, as purification goes on, becomes less soluble in ethyl alcohol. It was finally purified from dilute alcohol. The cinobufotoxin is then obtained in clusters of fine needles which melt at 200° with decomposition. In spite of several efforts we have not obtained the crystals in absolutely colorless form. The crystals were found to be insoluble in water, ether, acetic ester, chloroform, and petroleum ether, more readily soluble in ethyl alcohol, and soluble in pyridine.

The Sakaguchi reaction was strongly positive. With acetic anhydride and concentrated sulfuric acid first a pink color is formed which quickly disappears and then a green color develops. This color reaction is quite similar to that given by bufotoxin.

For analysis the substance was dried at 100° in a high vacuum over P_2O_5.

4.639 mg. substance: 3.47 mg. H_2O, 10.880 mg. CO_2.
4.511 " " : 3.41 " " 10.540 " "
4.440 " " : 3.21 " " 10.390 " "
5.410 " " : 3.99 " " 12.720 " "
3.520 " " : 0.239 cc. N at 24° and 758 mm.
3.440 " " : 0.226 " " " 25.5° " 758 "

$C_{43}H_{64}O_{12}N_4$.	Calculated.	C 62.32,	H 7.73,	N 6.77.
	Found.	" 64.21,	" 8.41.	
	"	" 64.05,	" 8.50.	
	"	" 63.84,	" 8.09.	
	"	" 64.12,	" 8.25.	
	"			N 7.71.
	"			" 7.49.

In the introduction we have already mentioned that the obtained analytical figures do not agree with those calculated. It may be that our product was not absolutely pure, or that the empirical

formula for cinobufotoxin and also for cinobufagin may have to be revised. We hope soon to be able to settle this point satisfactorily when we have more cinobufotoxin and when we have accumulated more data concerning the chemical composition of cinobufagin.

Hydrolysis of Cinobufotoxin.

400 mg. of cinobufotoxin were heated with 5 cc. of ethyl alcohol and 10 cc. of dilute hydrochloric acid (4 cc. of H_2O and 6 cc. of concentrated HCl) for 6 hours in a boiling water bath. After being cooled the cinobufagien was filtered off and the filtrate evaporated to dryness on the water bath. Cinobufagien, a yellow substance, could not be obtained in crystalline form. The residue left after evaporation of the water solution was extracted several times with ether, which left after evaporation nearly pure suberic acid. After one recrystallization from water the acid showed the melting point of 140°; the mixed melting point with a preparation of suberic acid showed no depression.

For analysis the substance was dried in a desiccator over $CaCl_2$.

4.532 mg. substance: 3.27 mg. H_2O, 9.095 mg. CO_2.

$C_8H_{14}O_4$.	Calculated.	C 55.17, H 8.04.
	Found.	" 54.70, " 8.07.

The residue left after extraction with ether was dissolved in a little water, flavianic acid was added, and the whole heated on a water bath until a clear solution resulted, put in the ice box, and let stand for 3 days. The precipitated flavianate of arginine was purified from water; it decomposed at 254°, and the mixed melting point with arginine flavianate showed no depression.

For analysis the substance was dried at 100°.

9.339 mg. substance: 4.547 mg. $BaSO_4$.

$C_6H_{14}O_2N_4 \cdot C_{10}H_4(NO_2)_2OHSO_3H$.	Calculated.	S 6.69.
	Found.	" 6.56.

Isolation of Cinobufagin.

The chloroform-ether solution obtained in the preparation of cinobufotoxin and containing the bufagin and other organic constituents of the dried venom was evaporated to dryness. The residue was dissolved in as little alcohol as possible, to the alco-

holic solution ether was added until the solution became light brown, and the mixture was let stand overnight in the ice box. After pouring off the supernatant fluid from the dark precipitate which had formed, petroleum ether was added; this threw down a precipitate which sometimes was already semicrystalline. It is best to add the petroleum ether in portions, pouring off the fluid every time from the resulting precipitate. The last fractions obtained were generally much easier to crystallize. The precipitate was dissolved again in a little ethyl alcohol and anhydrous ether added. Quite often we obtained at this stage a precipitate already crystalline, which was then further purified by several crystallizations from a little alcohol. If the precipitate is still gummy one has to dissolve it again in a little absolute alcohol, add ether—about 3 times the volume of alcohol used—and then make fractional precipitations with petroleum ether. The last fractions obtained generally yielded crystalline material.

Another method employed by us was to extract the gummy residue left after evaporation of the chloroform-ether solution mentioned above with ether until the undissolved part gradually became semicrystalline. This ether solution was used in the preparation of the chlorine-containing substance. The undissolved part could then be further purified from a little ethyl alcohol. The cinobufagin was thus obtained after recrystallization from ethyl alcohol in stout prisms which melted at 222–223°. The mixed melting point of cinobufagin and bufagin, obtained from *Bufo marinus*, showed a marked depression. Cinobufagin is very soluble in chloroform, alcohol, and acetone, less soluble in ether, and very little soluble in petroleum ether and water. Cinobufagin gave all the color reactions which have already been recorded by the Japanese chemists (2, 3).

For analysis the substance was dried at 100° in a high vacuum over P_2O_5. Final purification from absolute alcohol:

5.150 mg.	substance:	3.58 mg.	H_2O,	13.260	CO_2.
5.178	" "	: 3.62	" "	13.320	"
4.607	" "	: 3.09	" "	11.815	"
4.833	" "	: 3.29	" "	12.455	"

Final purification from absolute alcohol, ether, and petroleum ether:

4.934 mg. substance:	3.37 mg. H_2O,	12.635 mg. CO_2.		
4.917 " "	: 3.33 " "	12.560 " "		
4.300 " "	: 2.95 " "	11.030 " "		
$C_{29}H_{38}O_7$.	Calculated.	C 69.88,	H 7.68.	
	Found.	" 70.27,	" 7.78.	
	"	" 70.17,	" 7.84.	
	"	" 69.95,	" 7.51.	
	"	" 70.27,	" 7.62.	
	"	" 69.84,	" 7.64.	
	"	" 69.68,	" 7.58.	
	"	" 69.96,	" 7.68.	

Acetylcinobufagin.

200 mg. of cinobufagin were heated with 4 cc. of acetic anhydride for 2 hours in a boiling water bath. After cooling, water was added and the solution let stand overnight. The acetyl derivative obtained after filtration was purified from dilute ethyl alcohol. Fine white needles which melted at 195–196° were obtained. The mixed melting point of acetylcinobufagin and acetylbufagin showed marked depression.

For analysis the substance was dried at 100° in a high vacuum over P_2O_5.

5.160 mg. substance:	3.54 mg. H_2O,	13.090 mg. CO_2.		
4.910 " "	: 3.37 " "	12.400 " "		
4.880 " "	: 3.29 " "	12.300 " "		
5.510 " "	: 3.67 " "	13.950 " "		
4.071 " "	: 2.74 " "	10.265 " "		
$C_{31}H_{40}O_8$.	Calculated.	C 68.90,	H 7.47.	
	Found.	" 69.18,	" 7.68.	
	"	" 69.04,	" 7.70.	
	"	" 68.75,	" 7.55.	
	"	" 69.05,	" 7.46.	
	"	" 68.77,	" 7.53.	

Cinobufaginic Acid.

0.3 gm. of cinobufagin was heated with an alcoholic solution of potassium hydroxide (0.5 cc. of concentrated KOH and 5 cc. of 96 per cent ethyl alcohol) for 3 hours on a water bath. After cooling and filtration, the filtrate was made slightly acid with hydrochloric acid. After standing overnight the precipitate was filtered off and dried at room temperature. The acid was slightly yellow and could not be obtained in crystalline form. For further

purification the acid was dissolved in ethyl alcohol, in which it is very soluble, and then water was gradually added until the solution stayed cloudy. The next day a precipitate which was slightly yellow had settled out. To the filtrate from this precipitate more water was added. This precipitate after filtration and washing with water was dried at room temperature and used for analysis. It was quite white in color.

The acid begins to decompose at 120°, and becomes more and more yellow until it is completely melted. Water is probably split off at higher temperatures. For analysis the substance was dried *in vacuo* over $CaCl_2$.

4.782 mg. substance: 3.52 mg. H_2O, 11.975 mg. CO_2.
4.715 " " : 3.43 " " 11.790 " "

$C_{27}H_{38}O_7$. Calculated.	C 68.35,	H 8.02.
Found.	" 68.28,	" 8.24.
"	" 68.23,	" 8.14.

Bufotalin Chloride (?)

Into the ether solution which was obtained in the preparation of cinobufagin (see under "Isolation of cinobufagin") hydrochloric acid gas was passed. After some time a thick brown oil, together with solid matter, was precipitated. The ether was poured off and the residue mixed well with water. After filtration and washing with water the precipitate was purified from 96 per cent alcohol in which it is only slightly soluble in the cold. One obtains colorless prisms which melt at 220–221°. The ether solution still contained quite an amount of organic matter which we are now investigating with the aim of isolating a derivative of cinobufagin from it. We found that the product obtained from pure cinobufagin and hydrochloric acid gas is very soluble in ether. The product obtained above is nearly insoluble in this solvent.

For analysis the substance was dried at 100° in a high vacuum over P_2O_5.

4.900 mg. substance: 3.48 mg. H_2O, 12.345 mg. CO_2.
4.776 " " : 3.29 " " 12.010 " "
3.292 " " : 0.265 " Cl.
3.163 " " : 0.260 " "

$C_{24}H_{33}O_4Cl$. Calculated.	C 68.50,	H 7.85,	Cl 8.43.
Found.	" 68.70,	" 7.95.	
"	" 68.31,	" 7.68.	
"			Cl 8.05.

SUMMARY.

From Ch'an Su, the dried venom of the Chinese toad the following compounds have been isolated so far:

1. Epinephrine (see reference (1)).
2. Cholesterol, which was identified as such.
3. Suberic acid, which was identified as such.
4. Another pressor principle, different from epinephrine, in the form of a flavianate. This principle seems to be closely related to or identical with the pressor principle isolated by Handovsky (9) from the European toad, *Bufo vulgaris.*
5. Cinobufotoxin, which seems to be chemically closely related to bufotoxin, isolated by Wieland and Alles (4) from the European toad, *Bufo vulgaris.* On hydrolysis with hydrochloric acid a decomposition product of cinobufagin which could not be crystallized, suberic acid, and arginine were obtained. The latter two compounds were identified as such.
6. Cinobufagin, which was found to be different from bufagin isolated by Abel and Macht from the tropical toad, *Bufo marinus.* Several reactions were carried out with cinobufagin in order to obtain information regarding its chemical composition.

It was found that the pharmacological action of cinobufotoxin and cinobufagin is quite similar to that of the digitalis glucosides.

From the mother liquor obtained in the preparation of cinobufagin, a chlorine-containing body was prepared. Contrary to Kotake (3), who assumed that this substance was derived from cinobufagin, we believe that it is a derivative of bufotalin. The latter has been isolated by Wieland and his coworkers (4, 7) from the European toad, *Bufo vulgaris.*

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CHEMICAL STUDIES ON TOAD POISONS.

III. THE SECRETION OF THE TROPICAL TOAD, BUFO MARINUS.*

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INTRODUCTION.

Two crystalline pharmacological agents have already been isolated from the secretion of the tropical toad (*Bufo marinus*) by Abel and Macht (1) who studied this species under the name of *Bufo agua*.[1] One of these two principles was found by them to be identical with epinephrine; the other, which was called bufagin, was found to resemble the digitalis glucosides in pharmacological action. We were interested in finding out if the secretion of this toad also contains a substance which is similar in its chemical composition to bufotoxin, isolated by Wieland and Alles (2) from the European toad, *Bufo vulgaris*, and to cinobufotoxin, obtained by Jensen and Chen (3) from Ch'an Su, the dried venom of the Chinese toad. We were successful in securing from the secretion of this toad a nitrogen-containing substance in crystalline form which seems to be chemically closely related to bufotoxin and cinobufotoxin and which we call marinobufotoxin. Unfortunately, we had only a very small amount of secretion at our disposal to work with, so that we did not obtain enough of this substance for a hydrolysis experiment but judging from the positive result of the Sakaguchi reaction one can assume that the molecule of marinobufotoxin also contains arginine as do bufotoxin and cinobufotoxin. It was found that the pharmacological action of marinobufotoxin is quite similar to that of cinobufotoxin. The analytical data

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[1] *Bufo marinus* is now the generally accepted name for this species.

obtained point to the following empirical formula for marinobufotoxin, $C_{42}H_{62}O_{11}N_4$, which is given with reservation.

At the same time bufagin was also isolated from the toad secretion and was found to be identical with a specimen of bufagin, prepared by Abel and Macht (1) and kindly given us by Professor Abel. The empirical formula $C_{28}H_{36}O_6$ is proposed for bufagin. On acetylation of bufagin with acetic anhydride a monoacetyl derivative was obtained, having the composition $C_{30}H_{38}O_7$. The empirical formulas for bufagin and acetylbufagin are given with reservation.

We hope soon to be able to secure a larger amount of the secretion of this toad and shall then study further the composition of marinobufotoxin and bufagin. Most of the analyses reported in this paper were carried out by Dr. Ing. A. Schoeller, Berlin-Schmargendorf, Germany.

EXPERIMENTAL.

Isolation of Marinobufotoxin.

We secured the fresh secretion[2] by squeezing the "parotoid glands" of the toad according to the directions given by Abel and Macht (1). The semifluid substance thus obtained was dried in an evacuated desiccator. The dried material was pulverized and extracted with 96 per cent ethyl alcohol at about 50°, until this solvent no longer took up any appreciable amount of material. The alcoholic extracts were then freed of alcohol by distillation under diminished pressure and the residue was mixed well with water until it became brittle. After filtration and drying at room temperature the material was extracted with a small amount of absolute ethyl alcohol at 50°. After filtration and cooling, ether was added to the filtrate until no more precipitate was formed and the mixture was allowed to stand overnight in the icebox. The next day more ether was added in order to make sure that there was no further formation of a precipitate. The ether throws down the marinobufotoxin, and most of the bufagin stays in solution. The precipitate is again treated with absolute ethyl alcohol and after filtration ether is added again. The marino-

[2] We would like to express our thanks to Miss Cochran of the Smithsonian Institution, Washington, for her help in obtaining the toad secretion.

bufotoxin gradually becomes less soluble in ethyl alcohol. It is finally purified from 80 per cent ethyl alcohol. The marinobufotoxin is thus obtained in clusters of fine needles which melt at 200° with decomposition. The crystals were found to be insoluble in water, ether, acetic ester, chloroform, and petroleum ether, more readily soluble in ethyl alcohol, and soluble in pyridine.

The Sakaguchi reaction was strongly positive. With acetic anhydride and concentrated sulfuric acid first a pink color is formed which quickly disappears and then a green color develops. This color reaction is quite similar to that given by bufotoxin and cinobufotoxin.

For analysis the substance was dried at 100° in a high vacuum over P_2O_5.

4.922 mg. substance: 3.52 mg. H_2O, 11.395 mg. CO_2.
4.942 " " : 3.52 " " 11.420 " "
3.160 " " : 0.210 cc. N at 22° and 770 mm.
3.118 " " : 0.208 " " " 22° " 770 "

$C_{42}H_{62}O_{11}N_4$.	Calculated.	C 63.16, H 7.77,	N 7.02.
	Found.	" 63.15, " 8.00.	
	"	" 63.03, " 7.97.	
	"		N 7.79.
	"		" 7.32.

Isolation of Bufagin.

The ethereal solution obtained in the preparation of marinobufotoxin and containing the bufagin is concentrated to a smaller volume, then petroleum ether is added until no more precipitate is formed, and the mixture is allowed to stand for 3 days in the ice box. The petroleum ether throws down bufagin which is dissolved again in a small volume of absolute ethyl alcohol, an equal volume of ether is added, and then petroleum ether until the solution stays slightly cloudy. The solution is put in the ice box, whereupon the bufagin generally comes out during the course of several hours in beautiful long needles which are purified from a little 90 per cent ethyl alcohol. The crystals melted at 212–213°. When they were mixed with a specimen of bufagin prepared by Abel and Macht, no depression in melting point could be observed. The properties of the bufagin prepared by us were found to be the same as given by Abel and Macht (1). The specimen of Abel and Macht was purified for analysis from absolute ethyl alcohol, ether, and

petroleum ether. For analysis the substance was dried at 100° in a high vacuum over P_2O_5.

4.730 mg.	substance:	3.44 mg.	H_2O,	12.455 mg.	CO_2.			
4.960 "	"	: 3.64 "	"	13.080 "	"			
5.655 "	"	: 4.06 "	"	14.905 "	"			
4.829 "	"	: 3.41 "	"	12.715 "	"			
		$C_{28}H_{36}O_6$.	Calculated.	C 71.80,	H 7.70.			
			Found.	" 71.84,	" 8.14.			
			"	" 71.93,	" 8.21.			
			"	" 71.89,	" 8.03.			
			"	" 71.81,	" 7.90.			

A specimen prepared by the authors was dried at 100° in a high vacuum over P_2O_5 and analyzed.

4.572 mg.	substance:	3.28 mg.	H_2O,	12.050 mg.	CO_2.
4.780 "	"	: 3.41 "	"	12.590 "	"
		$C_{28}H_{36}O_6$.	Calculated.	C 71.80,	H 7.70.
			Found.	" 71.89,	" 8.03.
			"	" 71.86,	" 7.99.

Acetylbufagin.

100 mg. of bufagin were heated with 2 cc. of acetic anhydride for 2 hours on a water bath. After the mixture was cooled water was added and the solution was allowed to stand overnight. The acetyl derivative, obtained by filtration, was purified from dilute ethyl alcohol. Fine needles which melted at 203–204° were obtained. For analysis the substance was dried at 100° in a high vacuum over P_2O_5.

4.423 mg.	substance:	3.10 mg.	H_2O,	11.370 mg.	CO_2.
4.872 "	"	: 3.42 "	"	12.530 "	"
4.900 "	"	: 3.36 "	"	12.640 "	"
4.831 "	"	: 3.30 "	"	12.470 "	"
		$C_{30}H_{38}O_7$.	Calculated.	C 70.70,	H 7.46.
			Found.	" 70.11,	" 7.85.
			"	" 70.14,	" 7.86.
			"	" 70.36,	" 7.67.
			"	" 70.39,	" 7.65.

SUMMARY.

From the secretion of the "paratoid glands" of the tropical toad, *Bufo marinus,* was isolated in crystalline form a nitrogen-contain-

ing substance which we named marinobufotoxin. This principle seems to be closely related chemically to bufotoxin and cinobufotoxin. The pharmacological action was found to be quite similar to that of cinobufotoxin. The elementary composition of the substance is represented by the formula $C_{42}H_{62}O_{11}N_4$.

Bufagin was also prepared and found to be identical with the bufagin isolated by Abel and Macht. It has the composition $C_{28}H_{36}O_6$.

A monoacetyl derivative of bufagin having the composition $C_{30}H_{38}O_7$ was prepared.

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TOTAL SUGAR OF BLOOD AND URINE.

III. THE REDUCING ACTION OF GLUTATHIONE.

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(Received for publication, January 17, 1930.)

In a recent paper (1) Benedict and Newton report the reducing action of glutathione upon the Folin-Wu blood sugar reagents as one-fifth that of glucose. According to these investigators glutathione may account for 10 to 20 mg. of the non-glucose reducing material in 100 cc. of blood. Since the hydrolyzable sugar of blood filtrate (2) might be an artifact, dependent upon an increased reducing action of glutathione after acid hydrolysis, the effects of this peptide have been studied in our laboratory in more detail.

Analytical Methods.

The methods, precautions, and corrections previously described (2–4) were used for these determinations. The newer modifications of the Folin and Folin-Wu reagents were adopted, together with the longer periods of heating (5), but the colors were generated by the use of 4 and 2 cc., respectively, of the Folin acid molybdate solution, and the colored solutions then diluted with water. These features of the older methods were retained because I was certain of the corrections to be applied. The phenol modification of the Sumner method for the determination of urine sugar was used, with 0.05 per cent solutions of glucose and glutathione, but the results were calculated to 0.01 per cent solutions for purposes of comparison. The modification of Benedict's method suggested by Everett (4) (the addition of 2 cc. of acid molybdate solution when diluting to 10 cc., and of 4 cc. when diluting to 25 cc.) was preferred, in order to prevent all danger of unequal fading. As noted previously, in Benedict's method the more dilute standards fade more rapidly, a fact which has made it impossible to deter-

mine the proportionality curve for 0.0005 to 0.0025 per cent solutions of glucose in this method.

Reducing Action of Glutathione.

Through the kindness of Doctor E. C. Kendall I was able to obtain the crystalline, tripeptide, or reduced form of glutathione (6) for these experiments. The sample was received in a sealed tube and freshly prepared solutions were used to determine the

TABLE I.

Reducing Action of Glutathione in Per Cent of Glucose Reduction.

	Folin-Wu.	Folin.	Benedict.	Sumner.
Glutathione.	(20–23)* 21	(10–12) 11	(2–3) 3	Approximate. 1
Glutathione after acid hydrolysis.	(22–26) 24	(10–12) 11	(3–5) 4	Approximate. 1
Glutathione + glucose.	(121–125) 123	(105–110) 108	(96–105) 100	(102–104) 103
Glutathione + glucose after acid hydrolysis.	(120–123) 121	(100–102) 101	(102–109) 104	(105–107) 106
Glutathione + glucose after alkaline hydrolysis.	(12–14) 13	(10–13) 12	(2–4) 3	

The concentration of glucose and of glutathione was 0.01 per cent in each case. 0.0025 per cent solutions of glucose were used as standards for the copper methods, and 0.025 per cent solutions for Sumner's method.

* Figures in parentheses represent the extreme deviations. Results have been corrected for blanks.

values given in Table I, which are averages of five to ten determinations. 0.01 per cent glutathione solutions were used, because they approximate the higher concentrations of glutathione in blood filtrates.

The Sumner and Benedict reagents are reduced only very slightly by glutathione under any of the experimental conditions, and these low values are not the result of fading. Since glutathione, by itself, gives practically no color in Benedict's method, one would

naturally expect a mixture of this substance with glucose to have the same reducing action as glucose alone. Benedict and Newton, however, interpreted their results as the disappearance of the reducing action of a non-sugar substance in the presence of glucose. The glucose equivalent by the Folin-Wu method agrees with that determined for glutathione by Benedict and Newton. The equivalent by the Folin method is approximately one-half of

TABLE II.

Effect of Adding Glutathione to Blood.

Reduction in terms of glucose (mg. per cent).

Sample No.	Method.	Original blood.		Blood + 100 mg. per cent glutathione.			Protein precipitant.
		Free sugar.	Hydrolyzable sugar.	Free sugar.		Hydrolyzable sugar.	
				Actual.	Theoretical.		
1	Folin-Wu.	98	14	118	119	6	Tungstic acid.
	Benedict.	82	17	86	85	12	" "
2	Folin-Wu.	84	14	106	105	9	" "
	Folin.	78	7	89	89	4	" "
	Benedict.	75	15	79	78	14	" "
3	Folin-Wu.	84	17	103	105	9	" "
	Folin.	78	7	87	89	2	" "
4	Folin-Wu.	120	14	118	120	12	Zinc sulfate.
	Folin.	114	7	114	114	9	" "
5	Folin-Wu.	80	12	81	80	11	" "
	Folin.	74	8	75	74	7	" "
6	Folin-Wu.*	78	7	98	99	7	" "
	"	95	14	118	116	11	Tungstic acid.
	Folin.	84	6	94	95	6	" "

The theoretical values were calculated from the data of Table I.

* In Sample 6 the glutathione was added to the deproteinized filtrates instead of to the original blood.

this value. Practically no change in reducing value occurred when the glutathione solutions, preserved with toluene, were allowed to stand for 24 hours. A second sample of the tripeptide, sent by Doctor Kendall, gave the same glucose equivalents, except that an average value of 19.5 per cent was obtained by the Folin-Wu method.

It is clear that acid hydrolysis, under the conditions of the total

sugar method of Everett and Shoemaker, causes no increased reduction of these copper reagents by glutathione. The peculiar decreases in reducing action, after acid hydrolysis of mixtures of glutathione and glucose with the Folin method, and after alkali hydrolysis with the Folin-Wu method, remain unexplained for the present. It is well known that the non-glucose reduction by Folin-Wu blood filtrates is often approximately the same with the Folin and the Benedict solutions, yet the response of these reagents to glutathione is essentially different. The exact nature of the non-glucose sugar, as determined by these methods, is therefore still uncertain.

Glutathione was also added to normal human blood before the precipitation of the proteins. In addition to the customary tungstic acid filtrates of such samples, several filtrates were made by Somogyi's method (7). In using this method, I substituted for the sodium hydroxide solution of Somogyi, an equivalent potassium hydroxide solution, carefully protected from glass. The reasons for this change have been given previously (2). From Table II it is evident that the tungstic acid filtrates contain all of the added glutathione, and that the reduction by this substance is merely added to that of the original blood. In samples to which glutathione is added, there is a tendency towards lower hydrolyzable sugar values. It has been our experience, in a series of unpublished experiments, that the zinc filtrates contain less free sugar, as previously shown by Somogyi, and that they also contain less hydrolyzable sugar. The precipitation by zinc of a portion of the hydrolyzable sugar together with the glutathione, must be regarded as merely fortuitous, since the addition of glutathione to blood leads to no increase in the hydrolyzable sugar values, but rather to decreases.

SUMMARY.

Glucose equivalents for glutathione have been determined. This peptide is not the source of the hydrolyzable sugar of blood, nor can it satisfactorily account for more than a trace of the free non-glucose sugar of blood, as determined by the Benedict and Sumner methods. Zinc precipitation of protein, by Somogyi's method, removes all added glutathione, and in addition, substances which constitute a part of the hydrolyzable sugar of blood.

Miss Fay Sheppard, Mr. Jess D. Herrmann, and Mr. John F. Kuhn, Jr. have generously assisted me in determining and checking the reported analyses.

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KINETICS OF THE REDUCTION OF CYSTINE AND RELATED DITHIO (R–S–S–R) ACIDS BY REVERSIBLE OXIDATION-REDUCTION SYSTEMS.*

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The reduction of cystine and related dithio acids (R-S-S-R) to thiol acids (R-S-H) presents an interesting phase in the study of the oxidation and reduction properties of the cysteine-cystine and other dithio-thiol acid systems.

Reaction rates of the reduction of these compounds by the reversible chromous-chromic and vanadous-vanadic systems, have been determined under various conditions of concentrations of reactants, acidity, temperature, and oxidation-reduction potentials of the reducing system.

Information of particular biochemical interest has been obtained regarding the possible mechanism of the reduction of R-S-S-R to R-S-H, the nature of the apparent reversibility of the system, and the effect of the constitution of the attached groups on the ease of reduction of R-S-S-R to R-S-H. Also of interest is the effect of the oxidation-reduction potential of the reagent on the rate of the reduction.

The "reduction" potentials of cysteine and other thiol compounds and their reaction towards various oxidizing agents have been extensively investigated in recent years but the "oxidation" potential and oxidizing properties of cystine and dithio acids (R-S-S-R) have received but little attention apparently for the reason that cystine and the other dithio compounds appeared to be relatively unreactive under the conditions studied.

Dixon and Quastel (7), Michaelis and Flexner (13), and Kendall

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and Nord (11), have demonstrated that the cystine (R-S-S-R) did not affect the potentials imposed by cysteine (R-S-H) on gold, platinum, or mercury electrodes. Kendall and Nord (11), Dixon and Tunnicliffe (8), and Kendall and Loewen (10), have also studied the potentials or oxidation reaction rates of R-S-H compounds in the presence of indigo disulfonate and other substances. Harrison (9) also has studied the rate of oxidation of R-S-H compounds in the presence of catalyzers and inhibitors.

Since the R-S-S-R—R-S-H system did not behave in a manner analogous to that of such mobile reversible organic systems as quinone-hydroquinone, though either component could be transformed into the other by suitable reagents, it was thought that an application of methods designed for the study of irreversible systems might be of advantage if applied to the heretofore neglected phase of the subject, the reduction of cystine and dithio acids (R-S-S-R).

Conant (3) suggested the application of such methods as devised by Conant and Lutz (5) and Conant and Cutter (4) for irreversible systems to the study of the cystine-cysteine system. The general principles of these methods, with extensive modification because of the peculiar nature of the dithio-thiol systems and the nature of the reagent systems, have been applied in the present work.

EXPERIMENTAL.

The reaction studied was the reduction of dithio acids (R-S-S-R) to thiol acids (R-S-H) by reversible reagents of known oxidation-reduction potential. Mixtures of chromous chloride and chromic chloride, or other systems, in excess and in such proportion as to be well poised, were allowed to react with the R-S-S-R compound in solutions maintained at nearly constant acidity by relatively high concentrations of hydrochloric acid. Commercial tank nitrogen, deoxygenated by passing over hot copper, was used to remove and exclude oxygen from the reaction mixture.

Samples of the mixture were removed at timed intervals and added to an excess of maleic acid or *p*-nitro-dimethyl-aniline. These substances oxidized the excess of chromous to chromic salt, forming products which do not react with the iodine. The R-S-H formed was then titrated with KIO_3 after addition of KI and starch to the solution. An estimation of the amount of R-S-S-R reduced was thus obtained.

The validity of the iodine value as an estimation of the R-S-S-R reduced to R-S-H is based on the well established methods of R-S-H determination by iodine titrations (1, 12) and the asymptotic approach of all rate curves to a value representing complete reduction of R-S-S-R to R-S-H (Fig. 1).

Chromous chloride was prepared by reduction of chromic chloride by zinc. Mossy zinc of high purity was added to standard chromic chloride solution containing a known quantity of standard hydrochloric acid. About 2 N initial acidity was required for rapid complete reduction. A large excess of zinc was necessary and the excess of acid was reduced by allowing the zinc to react with it until practically no more hydrogen was evolved. This entire process required from 3 to 12 hours. Oxygen was excluded by a stream of purified hydrogen or nitrogen and when the reaction was complete the solution was filtered through asbestos and preserved under purified nitrogen in a burette similar to that used by Clark and Cohen (2) for storing reduced solutions. When carefully prepared less than 1 per cent of chromic salt remained and no apparent decomposition, as determined by the color or by analysis for the chromous salt, took place over a period of several days. Blank determinations on typical rate experiments showed that no appreciable amount of chromous salt was lost by oxidation or spontaneous decomposition under the usual experimental conditions and time duration. A sufficiently close estimation of the chromous chloride and hydrochloric acid content was obtained by using standard solutions and weighing the zinc quantitatively before and after the reduction of chromic chloride. The vanadous chloride used in certain experiments was prepared in a manner similar to that of the chromous solutions, with ammonium vanadate as starting material.

The dithio (R-S-S-R) acids were prepared by the methods of Biilmann (1) which consist of a preparation of the thiol acid by a reaction of the corresponding halogen acid with potassium ethyl xanthate, followed by decomposition of the resulting complex by ammonium hydroxide, removal of the xanthogenate amide, and liberation of the thiol acid by hydrochloric acid. After extraction with ether and purification by fractional distillation, the thiol acid was converted into dithio acid by oxidation by iodine. Purification of the dithio acids was accomplished by reprecipitation from

alcohol and water-free ether solutions by petroleum ether or, when the acids were sufficiently insoluble in strong hydrochloric acid, by dissolving them in dilute sodium hydroxide and precipitating with acid. Standard solutions were made up by direct weighing or by standardization against sodium hydroxide. When the acids were not sufficiently soluble, salts were formed by dissolving them in slight excess of sodium carbonate solution. Cystine was prepared from hair by hydrolysis and purified by reprecipitation at proper acidity.

The reaction vessel consisted of a rubber stoppered glass cylinder which was immersed in a constant temperature oil bath maintained within 0.3° of the desired temperature.

Measured quantities of standard solutions of chromic chloride, hydrochloric acid, and dithio compound, and the proper amount of water, were put into the vessel and deoxygenated by passing purified nitrogen or hydrogen (hydrogen was shown not to affect the reactions) through the mixture for at least 30 minutes. A stream of nitrogen was also allowed to pass through the mixture during the reaction and a mercury seal prevented possible back flow of oxygen. The chromous chloride was then added and samples taken at timed intervals.

By pipetting the sample directly into an excess of maleic acid (10 cc. of 1 M) or *p*-nitro-dimethyl-aniline (1 gm. in 10 cc. of 6 M HCl) the excess chromous chloride was converted into chromic chloride and the reaction stopped.

The reaction of these substances was so fast that only a few per cent of chromous compound remained after the 1st minute, this reduction in concentration seemed sufficient to consider the chromous dithio reaction stopped. Blank determinations showed that essentially complete oxidation of chromous chloride had occurred within about 3 minutes for *p*-nitro-dimethyl-aniline and about 7 minutes for maleic acid.

When maleic acid was used a purple color resulted on formation of the chromic salt so the solution was well diluted before titration with the standard KIO_3 in presence of excess KI and starch as indicator. When *p*-nitro-dimethyl-aniline was used, it was necessary to remove the precipitated aniline base from the diluted solution by filtration. Fading end-points occurred in titrating cysteine or others of the thiol compounds so an end-point stable for 5 minutes

TABLE I.

Experimental Conditions and Calculated Velocity Constants of the Rates of Reduction of Dithio Acids (R–S–S–R) by Chromous Chloride and Vanadous Chloride.

Experiment No.	Dithio (s).	$CrCl_2$ (c).	$CrCl_3$ concentration.	HCl concentration.	$ZnCl_2$ concentration.	Reaction range selected.	No. of determinations in range selected.	k (ps. 1st ord.) Limits.	k (ps. 1st ord.) Mean.
				Dithiodiglycollic acid.					
	M	M	M	M	M	per cent			
1	0.0025	0.025	0.00625	1.00	0.043	27–78	6	0.015–0.012	0.014
2	0.005	0.05	0.0125	1.00	0.086	27–73	6	0.050–0.043	0.047
3	0.01	0.1	0.025	1.00	0.16	22–81	6	0.124–0.119	0.123
4	0.02	0.2	0.05	1.00	0.32	24–70	4	0.343–0.302	0.330
5	0.005	0.05	0.2	1.00	0.086	20–75	8	0.054–0.047	0.052
6	0.01	0.1	0.4	1.00	0.16	29–83	5	0.165–0.147	0.155
7	0.005	0.05	0.0125	0.50	0.115	23–77	6	0.121–0.104	0.116
8	0.005	0.05	0.0125	0.25	0.115	31–80	6	0.307–0.241	0.265
9*	0.02	0.2	0.05	1.00	0.478	24–78	5	0.278–0.253	0.260
				Dithiodilactic acid (Na_2 salt).					
10	0.01	0.1	0.025	1.00	0.234	21–80	7	0.029–0.022	0.025
11	0.02	0.2	0.05	1.00	0.478	22–78	6	0.060–0.054	0.057
12	0.01	0.1	0.4	1.00	0.239	27–78	6	0.026–0.020	0.023
13	0.01	0.1	0.025	0.50	0.239	27–76	7	0.077–0.060	0.069
14	0.02	0.2	0.05	1.00	0.860	17–78	7	0.059–0.053	0.057
15*	0.02	0.2	0.05	1.00	0.478	26–72	7	0.049–0.044	0.048
				Cystine.					
16	0.005	0.05	0.0125	0.50	0.154	26–72	5	0.015–0.017	0.0156
17	0.01	0.1	0.025	0.50	0.308	23–68	6	0.033–0.038	0.0332
18	0.02	0.2	0.05	0.50	0.460	25–67	7	0.070–0.088	0.0815
19	0.005	0.05	0.2	0.50	0.154	25–75	6	0.019–0.015	0.0170
20	0.01	0.1	0.4	0.50	0.308	28–67	7	0.040–0.028	0.0322
21	0.02	0.2	0.05	1.00	0.616	20–74	8	0.025–0.030	0.0277
22	0.02	0.2	0.05	0.50	0.616	24–75	6	0.063–0.097	0.0749
23	0.02	0.2	0.05	0.25	0.616	32–83	4	0.185–0.218	0.197
24*	0.02	0.2	0.05	0.25	0.478	17–67	6	0.096–0.093	0.092

* Temperature was 70° except in Experiments 9, 15, 24, and 31 in which cases the temperature was 60°.

TABLE I—*Concluded.*

Experiment No.	Dithio (s).	$CrCl_2$ (c).	$CrCl_3$ concentration.	HCl concentration.	$ZnCl_2$ concentration.	Reaction range selected.	No. of determinations in range selected.	k(ps. 1st ord.). Limits.	k(ps. 1st ord.). Mean.
Dithiodihydracrylic acid (Na_2 salt).									
	M	M	M	M	M	per cent			
25	0.01	0.1	0.025	1.00	0.308	14–42	5	0.0022–0.0017	0.0019
26	0.01	0.1	0.025	1.00	0.237	29–52	3	0.0016–0.0015	0.0016
27	0.02	0.2	0.05	1.00	0.616	20–67	4	0.0076–0.0072	0.0073
28	0.02	0.2	0.05	1.00	0.474	30–80	5	0.0071–0.0064	0.0069
29	0.02	0.2	0.05	0.50	0.474	22–63	5	0.0088–0.0081	0.0084
30	0.02	0.2	0.05	0.25	0.474	28–78	7	0.0179–0.0266	0.0231
31*	0.02	0.2	0.05	1.00	0.482	17–75	3	0.0023–0.0016	0.0016

Experiment No.	Dithio (s).	VCl_2 (c).	VCl_3 concentration.	HCl concentration.	$ZnCl_2$ concentration.	k(ps. 1st ord.) mean.
Dithiodiglycollic acid.						
	M	M	M	M	M	
51	0.02	0.2	0.02	1.0	1.0	0.00063
52	0.02	0.2	0.2	1.0	1.3	0.00055
53	0.02	0.2	0.02	0.5	1.0	0.0012
Cystine.						
54	0.05	0.2	0.02	1.0	1.0	0.00008
55	0.05	0.2	0.02	0.5	1.0	0.00032

was taken as representing complete reoxidation. No appreciable reaction takes place between the iodine and maleic acid or the nitro compound or their reduction products.

Although the sources of error in the outlined procedure are many, the amount of potassium iodate used seems to give a fairly consistent, reproducible index of the quantity of R-S-S-R reduced.

Results.

A study of the effects on the reaction rates of the reduction of R-S-S-R by the reversible oxidation-reduction systems, of the concentration of the reactants, the oxidation-reduction potential of the reversible systems, the acidity, the temperature, and their

chemical constitution has been made. Table I gives a summary of the conditions of the individual experiments. Only those determinations within the range of 20 to 80 per cent conversion have been considered sufficiently significant for use in calculations of the

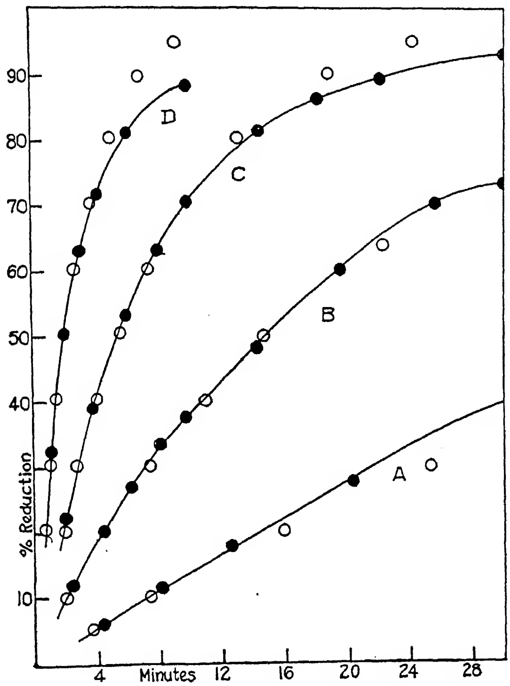

FIG. 1. The effect of the concentrations of the reactants on the rate of reduction of dithiodiglycollic acid by chromous chloride. Chromous chloride concentration was 10 times that of the dithio in each case. Concentration of dithiodiglycollic acid for Curve A, 0.0025 M; for Curve B, 0.005 M; for Curve C, 0.01 M; for Curve D, 0.02 M. Acidity 1 M HCl. Temperature 70°. ● Experimental results. ○ Theoretical calculations assuming a "pseudo first order" reaction.

reaction velocity constants and these only have been recorded. Groups of related experiments will be presented under their proper heading.

Order and Mechanism of the Reaction.

Preliminary experiments showed that the data would not accurately fit in the general equations for a first, second, or third order

TABLE II.

Calculation of Velocity Constants of the Reduction of Dithiodiglycollic Acid from the Results of Experiments 2, 3, and 4, Table I.

Experiment No.	Time.	Fraction converted.	$s - x$	Log $\frac{s}{s-x}$.	$k_{(\text{ps. 1st ord.})}$.	$k_{(\text{2nd ord.})}$.
	min.		*mols*			
2	0.00	0.000	0.005	0.000		
	2.25	0.119	0.00440	0.0550	0.0562	1.135
	4.25	0.197	0.00401	0.0948	0.0513	1.058
	6.25	0.270	0.00365	0.1367	(0.0503)	(1.023)
	8.25	0.336	0.00332	0.1775	(0.0497)	(1.008)
	10.25	0.375	0.00312	0.2041	(0.0458)	(0.942)
	14.25	0.478	0.00261	0.2769	(0.0448)	(0.936)
	22.25	0.636	0.00182	0.4385	(0.0454)	(0.942)
	30.25	0.730	0.00135	0.5682	(0.0433)	(0.906)
	40.25	0.810	0.00095	0.7210	0.0413	0.868
	50.25	0.847	0.00076	0.8149	0.0373	0.793
	60.25	0.878	0.00061	0.9138	0.0349	0.747
Mean of selected values in parentheses =					0.047	0.960
Under the conditions studied $\frac{k_{(\text{ps. 1st ord.})}}{c} = k_{(\text{2nd ord.})}$ =						0.94
3	0.00	0.000	0.01	0.0000		
	2.00	0.220	0.00780	0.1076	(0.124)	(1.255)
	4.00	0.393	0.00607	0.2172	(0.125)	(1.281)
	6.00	0.530	0.00470	0.3280	(0.127)	(1.297)
	8.00	0.634	0.00366	0.4362	(0.125)	(1.279)
	10.00	0.700	0.00300	0.5228	(0.120)	(1.260)
	14.00	0.812	0.00188	0.7267	(0.119)	(1.310)
	18.00	0.847	0.00143	0.8450	0.108	1.148
	22.00	0.885	0.00115	0.9390	0.098	1.047
	30.00	0.925	0.00075	1.124	0.086	0.923
	63.00	0.940	0.00060	1.223	0.045	0.885
Mean of selected values in parentheses =					0.123	1.260
$k_{(\text{ps. 1st ord.})}$ divided by (c) =						1.23
4	0.00	0.000	0.02	0.000		
	1.12	0.321	0.01357	0.167	(0.343)	(1.760)
	2.08	0.505	0.00989	0.306	(0.349)	(1.740)
	3.10	0.632	0.00735	0.435	(0.323)	(1.658)
	4.06	0.711	0.00578	0.539	(0.302)	(1.595)
	6.05	0.815	0.00370	0.732	0.279	1.473
	8.08	0.865	0.00270	0.870	0.248	1.103
	10.12	0.871	0.00257	0.891	0.203	1.078
	18.16	0.891	0.00218	0.995	0.126	1.688
Mean of selected values in parentheses =					0.326	1.688
$k_{(\text{ps. 1st ord.})}$ divided by (c) =						1.63

reaction. To obtain the advantage of simplified forms of these equations, the molar concentration of chromous chloride (c) at the beginning of the reaction was always made 10 times that of the dithio compound (s). When this was done the data of individual experiments, expressed graphically, showed curves resembling first order reaction curves (Fig. 1), which result is that usually obtained when the concentration of one reactant is high in respect to that of the other. Since k varies with the concentration of chromous chloride (c), k is not a true, but only a "pseudo" first order constant. A calculation of the "pseudo first order" constant (14) which may be expressed

$$k_{(\text{ps. 1st ord.})} = \frac{2.3}{t} \log_{10} \frac{s}{s - x}$$

showed that it varied only from 10 to 20 per cent for the range of 20 to 80 per cent reaction (Table II).

The second order constant,

$$k_{(\text{2nd ord.})} = \frac{2.3}{t(c - s)} \log_{10} \frac{s(c - x)}{c(s - x)}$$

calculated for three different original concentrations of chromous chloride and dithio compound (Table II), indicated that the reaction was not precisely second order, since the three mean values are not sufficiently close; 0.94, 1.23, 1.63.

The pseudo first order constant, $k_{(\text{ps. 1st ord.})}$, obtained in the manner described is related to the true constant, $k_{(n\text{th ord.})}$, according to the equation,

$$k_{(n\text{th ord.})} = \frac{k_{(\text{ps. 1st ord.})}}{(c)^{n-1}},$$

when the ratio $(c):(s)$ is ten or greater (14). A calculation of $k_{(n\text{th ord.})}$ shows the value of n to be approximately 2.3 to 2.4 for all of the dithio compounds studied.

Since the order of reaction so obtained is not integral, the pseudo first order constant was used for comparing results of various series of experiments in which only one factor was allowed to vary.

On the assumption that the reaction is of the second order, or approximately so, many attractive mechanisms might be depicted,

e Aqueous Buffer Solutions, Showing Their Relative Degree of Mobility.

Dithio $\underset{-2H^+ - 2e}{\overset{+2H^+ + 2e}{\rightleftarrows}}$ 2 Thiol (R—S—S—R) 2(R—S—H)	Aldehyde $\underset{-2H^+ + 2e}{\overset{+2H^+ + 2e}{\rightleftarrows}}$ Alcohol ($CH_3C(H){=}O$) (CH_3CH_2OH)
1. Either substance can be converted nto the other by properly selected mobile eversible oxidation-reduction systems.	1. Either substance can be converted into the other (in aqueous solution) only by the action of very powerful reagents.
2. No equilibrium is attained within easonable time. 3. The potentials of reversible oxidation-eduction systems which can cause measur-ible reduction of R–S–S–R usually are about 00 to 400 mv. more negative than the po-ential of the systems which can cause the xidation of R–S–H under comparable onditions.	2. No equilibrium is attained within reasonable time in aqueous solutions. 3. Oxidation occurs at a measurable rate only when powerful reagents (H_2CrO_4) are employed. Reduction occurs at a measurable rate only when powerful reagents (sodium amalgam in the absence of water) are employed.
4. The reduction of R–S–S–R by mobile reversible systems is measurable and is apparently bimolecular. The oxidation of R–S–H is greatly influenced by catalysis by traces of metals so that the rate measurements are uncertain. The reduction rate of R–S–S–R is not affected by changes in the imposed potential caused by altering the ratio of oxidant to reductant of the reagent system. A change to a less negative reagent system lowers the rate of the reduction.	4. No published information appears to be available which demonstrates the reaction rates of this system with mobile reversible reagent systems.
5. No equilibrium potentials are obtained after reasonable time. The potentials obtained experimentally appear to be independent of the R–S–S–R concentration but are affected by the R–S–H concentration.	5. No significant potentials are measurable.
6. A molecule of R–S–S–R is converted into 2 molecules of R–S–H on reduction.	6. No rupture of the molecule results in conversion of either form into the other.

however, reaction rates do not necessarily form a safe basis for indicating the mechanism of a reaction, but may be very useful in excluding mechanisms proposed when these are not consistent with the results of the kinetic studies.

Some mechanisms which have at times been proposed are based on a first order or on a third order reaction for the rate-controlling step. These may now be ruled out as depicting the predominating reactions. Thus may be excluded the possibility that R-S-S-R rapidly combines with a reductant to form a critical complex which subsequently slowly decomposes according to a first order reaction rate. In a similar way may be eliminated the mechanism involving a slow third order reaction between 1 molecule of R-S-S-R and 2 of reductant, followed by rapid decomposition of the critical complex into 2 R-S-H.

Question of Reversibility of Dithio-Thiol System.

The nature of the apparent reversibility of the dithio-thiol systems is of great importance in the study of biochemical reactions because of the wide-spread occurrence of this system, particularly as glutathione, in biological material and its possible participation in respiratory mechanisms.

One of the chief difficulties in classifying this reaction appears to lie in the definition of the term reversible. The difference between a reaction termed reversible and one termed irreversible may only be in the apparent difference in the rate at which they proceed. That the change from R-S-S-R to R-S-H is, in a certain sense, reversible is shown by the ease with which R-S-S-R can be quantitatively reduced to R-S-H by metallic tin in hydrochloric acid solution or R-S-H quantitatively oxidized to R-S-S-R in acid solution by iodine. That R-S-S-R can also be reduced to R-S-H by soluble labile reversible systems of known oxidation-reduction potential has been demonstrated by the data presented in this paper.

The nature of the reversibility may perhaps be best illustrated by a comparison to other systems as is made in the accompanying tabulation.

From this comparison it may be noted that the R-S-S-R—R-S-H system does not closely resemble the labile reversible systems nor the irreversible reactions whose rates are dependent upon a labile

reversible intermediate step. It resembles but slightly those exceedingly resistant changes illustrated by the aldehyde-alcohol systems.

In general, to avoid confusing this and similarly behaving systems with those demonstrably reversible systems such as quinone-hydroquinone, it is perhaps best to consider the changes involved in the system R-S-S-R—R-S-H as being irreversible; this conception being based on the definition of a reversible system as one whose components can be experimentally demonstrated to arrive at an equilibrium. A system then would be termed irreversible until proved reversible.

Effect of Potential of Reversible Reagent System on the Rate of Reduction of R-S-S-R.

Whereas the R-S-S-R—R-S-H system exhibits neither the properties of a labile reversible system nor those of an irreversible reaction dependent on a reversible step in the series, it appears to depend, to a certain extent, on the order of the potential of the reversible system whose reductant it oxidizes.

By altering the ratio of chromous to chromic concentration from (4:1) to (4:16) a rise in potential of about 70 millivolts results and similarly a change in ratio of concentration (10:1) to (1:1) for the vanadous-vanadic system causes a large shift in potential to the positive side. In neither instance (Table III) is the value of $k_{\text{(ps. 1st ord.)}}$ significantly affected. By changing the potential of the reagent by using the vanadous-vanadic system instead of the chromous-chromic system, a large decrease in rate resulted.

Queerly enough, although the reduction of R-S-S-R in general did not exhibit the properties of an irreversible reduction dependent on a reversible step, the result of a decrease in the reaction rate, produced in using a lower potential caused by changing systems, approximately agreed with the decrease in rate calculated by a loose interpretation of Conant's formulæ (3).

It might be well to mention at this point that had the usual procedure of Conant (3), that of employing only equal mixtures of oxidant and reductant and depending on changes to different systems to obtain different potentials, been applied to this unusually behaving reduction of R-S-S-R, the erroneous conclusion, that a reversible step entered in the reduction to R-S-H, might

TABLE III.

Effect of Potential of Reagent System on Reaction Rates.

*A. Chromous Chloride.**

$CrCl_2$:dithio acid ratio 10 M:1 M; temperature 70°; acidity and concentrations of $CrCl_2$ and dithio acid constant in each pair of experiments.

Experiment No.	Substance.	k(ps. 1st ord.) $\frac{CrCl_2}{CrCl_3} = \frac{4}{1}$ ($E_h = -0.435$)	k(ps. 1st ord.) $\frac{CrCl_2}{CrCl_3} = \frac{4}{16}$ ($E_h = -0.365$)
2, 5	Dithiodiglycollic acid (0.0025 M).	0.047	0.052
3, 6	" " (0.005 ").	0.123	0.155
10, 12	Dithiodilactic acid (0.01 M).	0.023	0.025
16, 19	Cystine (0.005 M).	0.015	0.017
17, 20	" (0.01 ").	0.033	0.032

B. Vanadous Chloride.

Substance.	HCl concentration.	k(ps. 1st ord.) $\frac{VCl_2}{VCl_3} = \frac{10}{1}$	k(ps. 1st ord.) $\frac{VCl_2}{VCl_3} = \frac{1}{1}$
	M		
Dithiodiglycollic acid.	1.0	0.00063	0.00055
(Similar $CrCl_2$ experiment.)		(0.33000)	(0.33000)
	0.5	0.0012	
" " "		(0.7000)	
Cystine.	1.0	0.00008 (approx.)	
(Similar $CrCl_2$ experiment.)		(0.02800)	
	0.5	0.00032 (approx.)	
" " "		(0.07500)	

* Similar results at different acidities, Table I.

TABLE IV.

Acidity or pH Effect.

Temperature 70°; $CrCl_2$:$CrCl_3$ ratio 4:1; $CrCl_2$:dithio acid ratio 10 M:1 M.

Substance.	R-S-S-R concentration.	k(ps. 1st ord.) in solutions of: 0.25 M HCl.	0.5 M HCl.	1.0 M HCl.
	M			
Dithiodiglycollic acid.............	0.005	0.265	0.116	0.047
Dithiodilactic acid................	0.01		0.069	0.025
Cystine..........................	0.02	0.197	0.075	0.028
Dithiodihydracrylic acid..........	0.02	0.0231	0.0084	0.0069

have been made. For those systems previously studied by Conant (3) such methods were adequate and the conclusions made were substantiated by collateral evidence, however, in attacking new systems by this method the effect of the change in potential of the reversible reagent system should be tested both by altering the oxidant-reductant ratio and by changing systems.

The reduction reaction of R-S-S-R to R-S-H by reversible systems apparently does not pass through a reversible intermediate.

Effect of Acidity on Reduction Rate.

For all of the R-S-S-R compounds studied, the rate of their reduction increases with a decrease in acidity (Table IV).

Because of the complexity of the reaction mixture it is difficult to decide on which factor or factors might be responsible for these differences; however, a few of the possibilities, such as the activity of the reagent reductant, complex ion formations, and the ionizations of the R-S-S-R acids, might be mentioned.

Temperature Coefficients of Reduction Reactions.

A marked difference in the temperature coefficients, determined for 60 and 70°, was found for the R-S-S-R compounds studied (Table V). The calculations (14) of the temperature coefficient from the Arrhenius Equation,

$$Q = \frac{R}{\left(\frac{1}{T_2} - \frac{1}{T_1}\right)} \log_e \frac{k_1}{k_2} = 52{,}000 \log \frac{k_{70}}{k_{60}}$$

form a basis for comparison.

Since certain of these Q values seem so unusual, and since the magnitude of the possible errors in certain of the cases is unknown, the investigation of them is being continued and, while no definite conclusions should be drawn from the meager data presented, the apparent effects of the constitution of the molecule on the temperature coefficient is of interest. Both acids having the -COOH attached to the same carbon atom to which the -S-S- is attached have low coefficients. Those acids whose -COOH is more removed have higher Q values. The value of the $-NH_2$-containing cystine is lower than that of the corresponding unsubstituted acid, showing

that the $-NH_2$ has somewhat the same tendency of lowering the Q value of the reduction of -S-S- when situated in close proximity.

TABLE V.

Temperature Effects.

$CrCl_2:CrCl_3$ ratio 10:1; $CrCl_2$:dithio acid ratio 10 M:1 M.

Temperature.	Substance.	(-S-S-) concentration.	HCl concentration.	$k_{\text{(ps. 1st ord.)}}$	Q
°C.		M	M		
60	Dithiodiglycollic acid.	0.02	1.00	0.260	5,400
70	" "	0.02	1.00	0.330	
60	Dithiodilactic acid.	0.02	1.00	0.048	3,900
70	" "	0.02	1.00	0.057	
60	Cystine.	0.02	0.50	0.0923	17,000
70	"	0.02	0.50	0.197	
60	Dithiodihydracrylic acid.	0.02	1.00	0.0016	33,000
70	" "	0.02	1.00	0.0069	

TABLE VI.

Chemical Constitution.

$CrCl_2:CrCl_3$ ratio 4:1; $CrCl_2$:dithio acid ratio 10:1; temperature 70°; 1 M HCl; and 0.2 M dithio acid.

	$k_{\text{(ps. 1st ord.)}}$	Approximate ratio of rates based on lowest.
Dithiodiglycollic acid. $(-S\ CH_2\ COOH)_2$	0.330	46
Dithiodilactic acid. $(-S\ CH(CH_3)\cdot COOH)_2$	0.057	8
Cystine. $(-S\ CH_2\cdot CH(NH_2)\cdot COOH)_2$	0.028	4
Dithiodihydracrylic acid. $(-S\ CH_2\cdot CH_2\cdot COOH)_2$	0.0072	1

Constitution of the R-S-S-R Molecule and its Effect on Reduction Reaction Rates.

While data on a sufficiently large number of R-S-S-R compounds have not been determined so that the general effects of substitution

on the rate of reduction of R-S-S-R cannot be outlined, those obtained are of interest since they show the direction of the influence of certain groups (Table VI).

The position of the -COOH group has a marked effect on the reduction of -S-S-; the rate decreases as -COOH is farther removed. The $-NH_2$ group shows a tendency to increase the reduction rate above that of the unsubstituted acid. A comparison of the two α-carboxylic acids reveals that the $-CH_3$ group decreases the reduction rate.

The acids showing greatest ease of reduction show also the lowest Q value for temperature coefficients and as the Q increases the ease of reduction apparently decreases.

The author wishes to thank Professor J. B. Conant for his many valuable suggestions and criticisms and wishes to express appreciation for his constant interest in the work.

SUMMARY.

Dithio (R-S-S-R) acids were reduced by reversible oxidation-reduction systems of high reducing intensity and reaction rates were obtained which resembled approximately those of second order reactions. These results serve as a criterion for the exclusion of certain reduction reactions as the predominating mechanisms.

The reduction rates differed for the various acids studied and seemed to depend on the position and nature of the groups attached to the -S-S- linkage, the rates being higher in those cases in which the -COOH or a $-NH_2$ group was adjacent to the -S-S- linkage. The reduction rates decreased as the acidity was increased. Temperature coefficients calculated for certain of the reactions were rather unusual and certain unexpected results were obtained for the effect on the reaction rates of the potential of the reversible reagent employed for the reduction; the rate of reduction was not affected by an alteration of the potential of the reagent employed when the oxidant-reductant ratio was changed, but the use of another system of different E_0 value did alter the rate of the reduction. Investigation of these various factors is being continued and is being extended to include such biologically important -S-S- systems as glutathione.

The results of this study of the reduction of R-S-S-R by reversible

reagents has led to certain conclusions regarding the possible nature of the apparent reversibility of the change R-S-S-R to R-S-H and a comparison of this system to other oxidation and reduction systems has been presented to assist in classifying this reaction.

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PROCEEDINGS OF THE AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS.

Twenty-Fourth Annual Meeting.

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THE MECHANISM OF CONVULSIONS IN INSULIN HYPOGLYCEMIA.

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The recent use of dehydration therapy in epilepsy[1] has restored interest in the observation of Drabkin and Shilkret[2] that water-starved dogs do not have convulsions during severe insulin hypoglycemia.

The condition of the blood and cerebrospinal fluid pressure has been studied in dogs, following injection of massive doses (20 units per kilo of body weight) of insulin. Continuous cisternal pressure readings were taken over periods of 3 hours or longer.

Dogs deprived of water for 5 days before the administration of insulin did not have convulsions. The cerebrospinal fluid pressure of this group of animals was either unchanged, rose slightly, or fell during the development of hypoglycemia and the accompanying anhydremia. The blood pressure in one dehydrated dog fell markedly during the 1st hour after insulin administration. Dogs given water by stomach tube for a comparable length of time invariably had convulsions during the hypoglycemia period. With the fall in blood sugar and rise in blood concentration the cerebrospinal fluid pressure of this group of animals rose markedly. The blood pressure was well maintained in this group.

The intravenous administration of hypertonic solutions of so called sodium arabinate to hydrated dogs in severe insulin convulsions resulted in a temporary return of the concentration of the blood towards normal. During this period (30 to 45 minutes) convulsions disappeared although severe hypoglycemia was still present. Hypoglycemia *per se* is not the cause of the convulsions. Both dehydration and intravenous injection of sodium

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[2] Drabkin, D. L., and Shilkret, H., *Am. J. Physiol.*, **83**, 141 (1927).

arabinate apparently interrupt the cycle: hypoglycemia → anhydremia → rise in cerebrospinal fluid pressure → convulsions; although other factors may be present.

In four preliminary experiments upon three hydrated animals from which both stellate ganglia had been previously removed insulin not only failed to produce convulsions but the hypoglycemic response was appreciably delayed and its magnitude greatly reduced. Anhydremia did not occur in these animals, nor was there a rise in cerebrospinal fluid pressure. Wormwood oil, however, produced convulsions in these dogs. On the other hand, the response of a normal hydrated animal to insulin was obtained in the cases of two dogs, one of which had previously had both splanchnic nerves resected and another in which both stellate ganglia had been removed as well as the splanchnics.

THE EFFECT OF CALCIUM CITRATE AND CARBONATE UPON THE EVACUATION OF A PROTEIN FROM THE STOMACH AND THE pH OF THE CONTENTS.

By WALTER C. RUSSELL and FRANCIS G. McDONALD.

(From the Department of Agricultural Biochemistry, New Jersey Agricultural Experiment Station, New Brunswick.)

The extent of the evacuation of coagulated egg white from the stomach of the albino rat and the pH of the contents were determined in the presence and absence of two levels of calcium citrate and calcium carbonate. In the case of the higher level each calcium compound supplied 2.1 per cent of calcium in the diets and at the lower level, 0.4 per cent. The evacuation was greatest in the absence of the calcium compounds, both calcium citrate and calcium carbonate reducing the amount of food that disappeared from the stomach when fed either at a high or low level.

When coagulated egg white was fed without the addition of a calcium compound the pH of the contents was 5.3. Upon incorporating 10 per cent and 1.917 per cent of calcium citrate in the food it was found that the higher level lowered the pH to 4.4 while the lower level had no effect. When calcium carbonate was introduced into the test meal instead of the citrate, in quantities sufficient to supply an equivalent quantity of calcium, it increased the pH of the contents to as great a value as 7.0. However, the smaller dosage of calcium carbonate raised the value only 0.6

pH over that of the control. The evacuation of the material from the stomach was approximately the same, on the average, when a pH of 7 was obtained, as a result of incorporating 5.2 per cent of calcium carbonate in the food, as when a pH of 4.4, induced by 10 per cent calcium citrate, was observed.

THE NUTRITIONAL REQUIREMENTS OF BROOK TROUT.

By C. M. McCAY.

(From the Animal Nutrition Laboratory, Cornell University, Ithaca.)

Growth curves of brook trout during a period of 30 months show marked contrast to those of the higher warm blooded species. Brook trout after long periods of stunting upon diets deficient in either calories or protein can resume normal growth under conditions of adequate feeding. Trout are able to utilize cooked starch better than raw starch as a source of calories. Although trout have comparatively short intestinal tracts they are able to tolerate relatively large amounts of cellulose without injury to their growth rates. In a series of studies in which various plant products have been used as sources of energy and protein it has been found that cottonseed meal is well utilized while linseed meal is an active poison. In a comparative study of various organs as foods it has been found that beef lungs as a sole article of diet are satisfactory during a period of 6 months. For the first time we have been able to show the need of brook trout for one or more of the recognized vitamins in yeast and cod liver oil.

A PRELIMINARY REPORT ON THE BASAL METABOLISM AND THE CALORIC AND PROTEIN INTAKE OF AMERICAN BORN CHINESE CHILDREN.

By CHI CHE WANG and JEAN E. HAWKS.

(From the Nelson Morris Memorial Institute for Medical Research, Michael Reese Hospital, Chicago.)

The data herein presented cover a part of a metabolic study conducted on twenty-one Chinese children, eleven boys between the ages of 5 and 11, and ten girls ranging from 5 to 17 years of age. With the exception of three girls including the oldest one they were all born and raised in Chicago. However, there was little difference between their food habits and those of their sisters and

brothers in China. All the boys except one and 50 per cent of the girls were slightly underweight and of all the children only two boys were up to the height standard according to the Baldwin-Wood tables. The averages of percentage deviation were 5.0 per cent underweight and 7.3 per cent underweight for the boys and 1.8 and 8.9 for the girls.

Basal metabolism was measured by the Tissot gasometer in connection with the Haldane gas analysis apparatus. The Benedict adiabetic bomb calorimeter was employed for the determination of the caloric value of the diet and the usual Kjeldahl method was used for the nitrogen.

The average total basal heat production was 1044 calories per 24 hours for the boys and 1024 for the girls. The corresponding values based on weight, height, or square meter of body surface of the two groups were 49.2 and 47.3 calories per kilo, 8.69 and 8.60 per cm., and 50.4 and 49.8 calories per square meter of body surface. In comparing our results with various standards we found that with the exception of the 17 year old girl whose metabolism was within the normal range, all our children had a higher basal heat production than the Benedict-Talbot standards whether the comparison was based on age, weight, height, or surface area and whether the values were expressed as total heat production per 24 hours, calories per kilo of body weight, or calories per square meter of body surface. Out of 220 comparisons made, only in three instances was the negative sign used. Of the nine possible comparisons made with Benedict-Talbot standards the one corresponding most closely was the value calculated for the total calories per 24 hours referred to age and the greatest variation occurred in the calories per kilo referred to age. The average percentage of deviation from the former was +6.8 for the boys and +11.5 for the girls, while the differences from the latter were +25.0 and +26.2 respectively. Our results were very close to both the Bailey and Dreyer standards with an average percentage of deviation from the former of −2.1 for the boys and +3.6 for the girls and from the latter standard were −4.0 and +3.4.

The daily caloric intake varied greatly in the same individual. However, with the exception of two extremes of the boys and the 17 year old girl the values ranged from 66.6 to 98.2 calories per kilo for the boys and 63.6 to 80.8 for the girls. In comparison with the standards of caloric requirement our values were closer

to those of Holt per kilo per 24 hours than either to his total per 24 hours or to the Hawley standard. The average percentage of deviation per kilo from the Holt standard was +0.4 for the boys and −8.4 for the girls.

The excess of caloric intake over the basal heat production varied somewhat with the financial circumstances as well as the education of the families. The values ranged from 14.6 per cent over the basal in a 5 year old boy with almost no activity to 125.1 per cent in an especially active 9 year old boy. The average percentage excess was 65.9 for the boys and 53.0 for the girls.

The results of our study of the children have failed to substantiate the general assumption that orientals are vegetarians. With the exception of one boy and the 17 year old girl who had slightly less than 2 gm. of protein per kilo of body weight, all the children had over 2 gm. with an average of 2.99 for the boys and 2.55 for the girls. However, in comparison with the Holt standard per kilo per 24 hours the average percentage of deviation was −4.8 for the boys and +1.2 for the girls.

In spite of the great variety of mixtures and soups used in their diet the dried food per kilo of body weight was surprizingly constant with an average of 16.2 gm. for the boys and 14.7 gm. for the girls. In general, fresh native vegetables predominated over those used in the United States and dairy products were sparingly included in their diet.

CARBOHYDRATE, VOLATILE FATTY ACIDS, WATER RETENTION, AND CLINICAL SYMPTOMS.

By J. F. McCLENDON.

(From the Laboratory of Physiological Chemistry, University of Minnesota, Minneapolis.)

A non-diabetic, given a diabetic diet, showed unstable blood sugar, low glucose and insulin (Lilly) tolerance, and high water retention on taking glucose. The micro method of Folin and Malmros for blood sugar was changed so that 0.02 cc. of blood and a 5 cc. graduated test-tube were used. A square jar of ferricyanide solution was used as a color filter. The blood volume was checked by the determination of the hemoglobin, with a Bausch and Lomb colorimeter and glass standard. Roughage had a

marked laxative effect on this patient, probably due to the fermentation products of the carbohydrate. Sugars which were quickly absorbed fermented little because they stayed only a short time in the gut, but when the blood sugar rose, fermentation took place, probably due to sugar diffusing from the blood to the gut: The following substances were found to be laxative: 50 gm. of wood flour (Cellu Flour), 50 gm. of India gum, 250 gm. of glucose (cerelose), 50 gm. of xylose (from Dr. Acree), 2 gm. of sodium formate (du Pont), 10 cc. of acetic acid, 5 cc. of lactic acid, 2 cc. of tributyrin. The xylose caused a rise in blood sugar from 135 to 250 mg. per 100 cc. During the 1st hour after xylose administration, 2.2 gm. were excreted, during the 2nd hour 1.4 gm., and for the next 5 hours 1.2 gm. per hour. After the administration of 250 gm. of glucose the blood sugar increased from 110 to 151 mg. in $\frac{1}{2}$ hour with no glycosuria. After the administration of 175 gm. of sucrose + lactose + starch, the blood sugar rose in 1 hour from 142 to 153 mg. The blood volumes were not an index of water retention but eating glucose caused water retention and very scanty urine. In 6 hours 500 gm. of carbohydrate and 1000 cc. of water were ingested and there were 315 cc. of water possible from the burning of the carbohydrate, and only 225 cc. were excreted while 300 cc. were lost in evaporation, with a retention of 790 cc. of water. It seemed probable that the water was stored in the skin but the edema test by intradermal injection of Ringer's solution was negative. White rats fed 2 weeks on 87.5 per cent starch, lactose, sucrose, maltose, or glucose, had about 60 per cent water content of the skin. The glucose tolerance of another non-diabetic given a diabetic diet was found to be low. It is suggested that xylose be used in reducing diets.

THE EFFECT OF FASTING AND CREATINE INGESTION UPON THE CREATINE AND NITROGEN CONTENT OF THE WHITE RAT.

By ALFRED CHANUTIN.

(From the Laboratory of Physiological Chemistry, University of Virginia, University.)

In the present investigation we have studied the creatine and nitrogen content of the whole rat (eviscerated) under various conditions. Previous work has shown that the per cent of creatine in the muscle of fasted animals is increased.

A more accurate indication of the creatine and nitrogen content of the organism may be obtained by expressing the results in terms of dry, fat-free tissue. It has been found that the creatine and nitrogen of dry, fat-free tissue remains constant during all stages of fasting. The percentage of these constituents is increased progressively, however, in the moist tissue. An analysis of the muscle of animals that had lost from 30 to 45 per cent of body weight shows an increase of 6 per cent in the creatine content. This increase does not manifest itself in the whole rat.

After feeding a creatine-containing diet, an increase of 12 per cent in the creatine content of the dry, fat-free tissue was noted. The nitrogen percentage was not changed.

These results indicate that the creatine percentage in the dry, fat-free tissue can be increased markedly. Fasting, however, has no effect on the constituents studied. These conclusions are rather striking in view of the many ideas and theories, expressed in the literature, that are based upon changes in creatine concentration of the organism as a result of fasting.

FURTHER STUDIES ON THE ORIGIN OF CREATINE.

By ERWIN BRAND, MEYER M. HARRIS, MARTA SANDBERG, AND MARGARET M. LASKER.

(*From the Laboratory Division and the Metabolic Department of the Medical Division, Montefiore Hospital, and the Department of Chemistry, New York State Psychiatric Institute and Hospital, Columbia-Presbyterian Medical Center, New York.*)

In a preliminary communication[3] it was reported that oral administration of glycine gives rise to an increased excretion of creatine in cases of progressive pseudohypertrophic muscular dystrophy. Further superimposition feeding experiments with gelatin and edestin show that a marked increase in creatine excretion is produced by the former and only a slight increase by the latter. From a consideration of the amount of glycine (and of arginine) present in the two proteins it would seem that this result is in agreement with our previous findings.

The increased creatine formation during fasting together with our results suggest the possible importance of tissues rich in

[3] Brand, E., Harris, M. M., Sandberg, M., and Ringer, A. I., *Am. J. Physiol.*, **90**, 296 (1929).

glycine (*e.g.* connective tissue, bone, etc.) as reservoirs of deposit or reserve protein. We intend to use this idea as a working hypothesis in the planning of further experiments.

CREATINE METABOLISM IN A CASE OF PROGRESSIVE MYOSITIS OSSIFICANS: A COMPARISON WITH GENERALIZED MYOSITIS FIBROSA.

BY MEYER BODANSKY AND EDWARD H. SCHWAB.

(*From the Laboratory of Biological Chemistry and the Department of Medicine, University of Texas School of Medicine, Galveston.*)

In a case of progressive myositis ossificans, in which nearly all of the muscles showed a greater or lesser degree of replacement by osseous tissue, creatinuria did not occur, either on a creatine-free diet or on the usual "house" diet which included a small amount of meat. The daily creatinine output, which was fairly constant, averaged 1.06 gm., the creatinine coefficient being 18.3. This observation is what might be expected in an individual whose active musculature is so definitely below normal. The ability to retain exogenous creatine, given as such or in the form of meat, was definitely below normal, but not as deficient as in the case of myositis fibrosa previously described. It is of interest to note that whereas myositis fibrosa is associated with a generalized subacute or chronic inflammatory process in the muscles, this is not the case in myositis ossificans. The greater ability to utilize creatine in the latter disease, despite the extensive involvement of the muscles, supports the view suggested on the basis of earlier work with myositis fibrosa that an inflammatory condition in the muscles interferes with the normal storage of creatine.

THE PRODUCTION OF KYNURENIC ACID FROM TRYPTOPHANE DERIVATIVES.

BY CLARENCE P. BERG.

(*From the Biochemistry Laboratory, State University of Iowa, Iowa City.*)

Berg, Rose, and Marvel[4] have recently shown that, whereas benzoylated tryptophane and the formaldehyde condensation product of the amino acid are unable to render a tryptophane-deficient basal diet adequate to support growth in young rats,

[4] Berg, C. P., Rose W. C., and Marvel, C. S., *J. Biol. Chem.*, **85**, 207 (1929).

On page xi, Vol. lxxxvii, No. 2, June, 1930, line 16, read *Acetyltryptophane, injected subcutaneously, is apparently not very readily converted to kynurenic acid* for *Acetyltryptophane, injected subcutaneously, is apparently not converted to kynurenic acid.*

both acetyltryptophane and tryptophane ethyl ester hydrochloride permit as good growth as tryptophane itself. Tryptophane ethyl ester hydrochloride could be shown to be split very readily *in vitro* by enzymes occurring in the alimentary tract, but no clear demonstration was secured that acetyltryptophane could be more than partially hydrolyzed even after 24 hour enzymolysis.

It seemed of interest to correlate these findings with determinations of kynurenic acid output following the administration of the derivatives to rabbits, inasmuch as this biological synthesis of kynurenic acid might lend an insight into *in vivo* cleavages. Injected subcutaneously, neither benzoyltryptophane nor the formaldehyde condensation product yields an appreciable amount of kynurenic acid; tryptophane ethyl ester hydrochloride alone seems to give rise to an output of kynurenic acid comparing favorably with that obtained after administering an equivalent amount of free tryptophane. Acetyltryptophane, injected subcutaneously, is apparently not converted to kynurenic acid. Further experiments are under way to determine whether oral administration, especially of the acetyl derivative, will cause the excretion of kynurenic acid in an amount comparable with that obtained after the administration of an equivalent weight of tryptophane.

A HITHERTO UNDESCRIBED INBORN (?) ERROR OF METABOLISM RELATED TO TYROSINE.

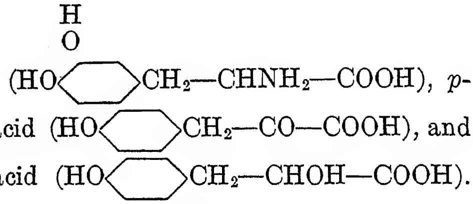
By GRACE MEDES.

(*From the Department of Medicine, University of Minnesota, Minneapolis.*)

The conceptions of tyrosine metabolism held by the investigator at the present time include three series of chemical reactions, the first two of which have been worked out in considerable detail while the last is based upon a few isolated observations and is more or less hypothetical. The two former are those reactions leading to the production of homogentisic acid and those ending in the formation of melanin through the agency of tyrosinase. So far, homogentisic acid has been found only in the alcaptonuric and the tyrosinase series exclusively in test-tube experiments and in plants and insects. I present what I believe to be a hitherto unrecognized inborn error of tyrosine metabolism, which demon-

strates the actual existence of the tyrosinase line in man, and which also throws light on the third line of reactions representing the normal catabolism of tyrosine.

2 years ago Berglund, Medes, and Lohmann reported our first attempts to isolate what we then believed to be a single reducing substance in a patient whose urine was found to reduce phosphomolybdate at room temperature in acid solution.

I have now isolated the following compounds: tyrosine, 3,4-dihydroxyphenylalanine  *p*-hydroxyphenylpyruvic acid, and *p*-hydroxyphenyllactic acid. The relative amounts of these compounds excreted have been estimated and will be given. The connection with melanin has been demonstrated since melanin has been isolated after the urine has been subjected to vigorous oxidation. In contrast to the condition in melanuria, neither melanin nor its immediate precursors are present in the untreated urine. In a case of melanuria which we have also had occasion to study, the compounds described in this paper are absent.

In isolating these compounds the urine was treated with lead acetate and the precipitate fractionated at pH of 5, 7, and 9 respectively. Fraction III contained the tyrosine, the other three compounds being in Fraction II. The pyruvic and lactic acid compounds were removed by continuous extraction with ether. On evaporating the ether completely the pyruvic and lactic acid compounds crystallized out. These were then taken up in a small volume of the water in which the lactic acid compound dissolved, the pyruvic acid compound being insoluble. The aqueous residue of Fraction II, after ether extraction, was saturated with SO_2 and evaporated to dryness at reduced pressure. The dried residue was dissolved in SO_2 water several times, boiled in purified charcoal, and reconcentrated in a desiccator over H_2SO_4 at reduced pressure. Dihydroxyphenylalanine finally crystallized out.

Both hydroxyphenylpyruvic acid and dihydroxyphenylalanine

reduce phosphomolybdic acid and the Folin copper sugar reagent, but at different rates and intensities. These differences were made use of in estimating the relative amounts of each present. A modification of Folin's tyrosine method was also employed since it determines all of the compounds except the dihydroxyphenylalanine.

For this condition I propose the name tyrosinosis rather than tyrosinuria; the latter would indicate simply loss of tyrosine as in cystinuria, whereas in this case there are excreted, in addition, metabolites representing at least two lines of metabolic processes.

The discussion of the theoretical significance of these findings can be merely touched upon now. More detailed considerations will be taken up after feeding experiments are performed.

THE RELATION BETWEEN THE INTAKE OF PROTEIN AND THE SYNTHESIS OF GLYCINE IN RATS.

By WENDELL H. GRIFFITH.

(*From the Department of Biological Chemistry, St. Louis University School of Medicine, St. Louis.*)

The rate of growth of young white rats was determined on diets containing sodium benzoate and 20, 25, 35, and 50 per cent of casein. The toxicity of these benzoate diets decreased as the intake of casein increased. Raising the protein from 20 to 35 per cent of the diet was more effective in decreasing the toxicity of the benzoate than raising the protein from 35 to 50 per cent of the diet. Diets containing casein which had been subjected to enzymatic hydrolysis *in vitro* were less toxic than similar diets containing untreated casein. In none of these experiments was the increase in the tolerance of the rats for benzoate proportional to the increase in the protein intake. It was concluded that rats do not readily synthesize glycine from the products of the metabolism of casein.

β-OXIDATION IN THE INDOLE SERIES.

By RICHARD W. JACKSON.

(*From the Laboratory of Physiological Chemistry, Yale University, New Haven.*)

In a recent paper,[5] the author presented evidence to show that out of a considerable series of compounds structurally related to tryptophane, indolepyruvic acid alone is able to assume the metabolic rôle of tryptophane. The question arises as to what is the fate of the derivatives other than the pyruvic acid. Indolepropionic and indolebutyric[6] acids have been studied. When indolebutyric acid was fed to albino rats, it was found possible to isolate approximately one-third of an equivalent of indoleacetic acid from the urine. This was identified by the melting point and the melting point of a mixture with a pure synthetic specimen. When the pure indoleacetic acid was fed, it likewise was isolated with approximately the same yield. Further, the subcutaneous injection of the sodium salt of indolebutyric acid into a rabbit resulted in approximately a 25 per cent yield of indoleacetic acid. Thus indolebutyric acid undergoes β-oxidation in the rat and in the rabbit. In addition to the indoleacetic acid, another indole acid was isolated; it seems highly likely that this material is indolecrotonic acid. Similar experiments with indolepropionic acid gave only a red amorphous dye. No crystalline material of any kind could be isolated. These also were the findings of Ward[7] who used a different fractionation scheme than that employed by the writer. The red dye most apparently contains an altered indole ring. The writer believes with certain reservations that no indolecarboxylic acid is formed in the metabolism of indolepropionic acid.

[5] Jackson, R. W., *J. Biol. Chem.*, **84**, 1 (1929). *Cf.* also Berg, C. P., Rose, W. C., and Marvel, C. S., *J. Biol. Chem.*, **85**, 207 (1929).

[6] The synthesis of this acid will be published shortly in collaboration with R. H. F. Manske of Yale University.

[7] Ward, F. W., *Biochem. J.*, **17**, 907 (1923).

CALCIUM AND PHOSPHORUS METABOLISM IN RELATION TO CERTAIN BONE DISEASES.

I. HYPERCALCURIA.

By GENEVIEVE STEARNS AND JULIAN D. BOYD.

(*From the Department of Pediatrics, College of Medicine, State University of Iowa, Iowa City.*)

Three patients have been studied, each of whom showed a definite increase in the amount of calcium excreted in the urine. The urinary phosphorus was also increased above the normal. Determinations of the serum calcium and phosphorus revealed the presence of a hypercalcemia in two of the patients, and a serum phosphorus consistently below normal in all. The bone conditions present in the three cases were diverse in nature, in contrast to the metabolic findings, which were very similar. Parathyroid exploration was performed on each patient, and the presence of a parathyroid adenoma was established in one. The excretion of calcium and phosphorus has been studied with varying intakes of these elements, both in these subjects and in normal controls for comparison.

THE DETERMINATION OF INORGANIC AND CONJUGATED SULFATES IN BIOLOGICAL FLUIDS.

By E. G. WAKEFIELD AND MARSCHELLE H. POWER.

(*From the Division of Medicine, The Mayo Foundation, Rochester, Minnesota.*)

Inorganic sulfates are precipitated with benzidine in the presence of trichloroacetic acid and acetone. Conjugated sulfates are precipitated similarly after hydrolysis with acid. The precipitate of benzidine sulfate is then oxidized with excess potassium dichromate in sulfuric acid, and the excess determined iodometrically. The oxidation of benzidine is quantitative, and the reaction affords a simple and precise method of determining the benzidine corresponding to amounts of sulfate ranging from 0.02 to 0.80 mg. of SO_4. The trichloroacetic acid filtrate from 3 cc. of serum is sufficient for the determination in duplicate of the serum inorganic sulfates.

COLLOIDAL URIC ACID.

By E. GORDON YOUNG and F. F. MUSGRAVE.

(From the Department of Biochemistry, Dalhousie University, Halifax, Canada.)

A study has been made of the conditions under which uric acid will form a gel in aqueous solution. The influence of supersaturation, of temperature, and of hydrogen ion concentration are all shown as important factors. The hydroxides of sodium, potassium, and lithium form with uric acid more or less stable gels within limiting conditions. This power of gel formation is also possessed by numerous organic bases, especially the lower alkyl amines, guanidine, hydrazine, coniine, hydroxylamine, and hexamethylenetetramine. Certain of these substances can also form sols with uric acid. The colloidal nature of the complex has been determined from diffusion experiments, molecular weight determinations, and microscopical observation. From quantitative estimations of solution ratios, of elementary composition and from the electrometric titration of pure uric acid, a theory is advanced to explain the colloidal behavior of uric acid in aqueous solution. Its applicability to normal physiology is demonstrated.

A COMPARISON OF BLOOD PROTEINS WITH THOSE OF NEPHRITIC URINE AND EDEMA FLUIDS.

By J. W. CAVETT.

(From the Biochemistry Laboratory, State University of Iowa, Iowa City.)

Proteins from urine, edema fluids, and blood plasma have been fractionated by ammonium sulfate. After fractionation they were heat-coagulated, freed of ammonium sulfate, and extracted with alcohol and ether to remove the lipids.

The proteins from the different sources were compared by racemization curves. These were obtained by following the optical rotations of 2 per cent solutions in 0.5 N alkali. The readings were made at 38° and extended over 12 days. The nitrogen distribution in these proteins was determined by a modification of the Van Slyke method.

The racemization curves and the nitrogen distribution indicate that the proteins from these different sources are identical or very similar.

FRACTIONATIONS OF THE TUBERCULIN PROTEIN.

By FLORENCE B. SEIBERT AND BETTY MUNDAY.

(From the Otho S. A. Sprague Memorial Institute and the Department of Pathology, The University of Chicago, Chicago.)

By filtering different tuberculins through graded membranes, and analyzing the fractions consisting of different sized molecules, it was possible to conclude that the constituent responsible for producing the tuberculin skin reaction and for giving a precipitin test in homologous immune sera is the protein and possibly only the very early cleavage products of the protein which have a very low molecular weight. It is probable that these early cleavage products are the chief causes of the toxicity of tuberculin, in both the normal and tuberculous animals. All possible effect of the polysaccharide has not been entirely ruled out. The polysaccharide is associated chiefly with these early cleavage products of the protein and can be removed by crystallization of the protein, or at a pH of 4.8. A tuberculin powder, containing 16 per cent of nitrogen, 2.7 per cent of polysaccharide, almost completely water-soluble and with a very high specific activity in the precipitin and skin tests (0.01 mg. produces a maximum skin reaction in tuberculous guinea pigs), and possessing very low primary toxicity, was made by half-saturating with $(NH_4)_2SO_4$ a raw tuberculin which had been concentrated by ultrafiltration.

CONCERNING THE CARBOHYDRATES ASSOCIATED WITH THE ETHER-SOLUBLE LIPOIDS OF TUBERCLE BACILLI.

By R. J. ANDERSON AND E. GILMAN ROBERTS.

(From the Department of Chemistry, Yale University, New Haven.)

The ether-soluble phosphatides isolated from the human and avian types of tubercle bacilli contain relatively large amounts of carbohydrates. After hydrolysis the phosphatide, Fraction A-3, from the human bacillus gave about 33 per cent of water-soluble material which contained glycerophosphoric acid and about equal parts of mannose, inosite, and a mixture of levorotatory reducing sugar which is probably similar to invert sugar. On treatment with phenylhydrazine this reducing sugar gave an osazone which was identical with glucosazone.

The phosphatide from the avian bacillus gave after hydrolysis

about 47 per cent of water-soluble material. In this mixture we have identified glycerophosphoric acid, mannose, inosite, and a reducing sugar which gave a glucosazone similar to the one from the human bacillus.

The so called wax isolated from a chloroform extract of the human bacillus was found to yield on hydrolysis about 40 per cent of water-soluble material, but the sugars contained in this fraction were quite different from those present in the phosphatides. No mannose could be isolated and inosite could be obtained only in minute traces. A relatively large proportion of a pentose was present which on treatment with diphenylhydrazine gave a hydrazone which was identical with the diphenylhydrazone prepared from *d*-arabinose. A very small amount of glucosamine was also obtained, but the major portion of the sugar mixture is as yet unidentified.

STUDIES ON GLUTELINS.

By FRANK A. CSONKA, M. J. HORN, and D. BREESE JONES.

(From the Protein and Nutrition Division, Bureau of Chemistry and Soils, United States Department of Agriculture, Washington.)

The characterization of our glutelin preparations has been made more definite by the determination of their specific rotation. We determined the racemization curves of α- and β-glutelins of wheat as well as that of gliadin. We found that the type of equation, $y = ax^b + c$, was applicable to the racemization curves of these three proteins. The calculated values agreed quite well with those found experimentally. The specific rotation of glutelins prepared from other cereals was also determined.

Wheat gluten dissolved in 0.5 M NaOH and incubated at 38° for 52 days shows approximately a 50 per cent drop in optical activity. The protein fractions which were separated from the incubated product were all optically active; none of them could, therefore, be called "racemic protein." All of these preparations lost their optical activity when they were heated with 20 per cent HCl or with 4 per cent NaOH. Alkali, therefore, at body temperature does not racemize proteins, but only produces some yet unknown intramolecular change in the cleavage products leaving presumably most of the asymmetric carbon atoms intact. These protein

products by hydrolysis with acid (or with alkali) at boiling temperature result in a hydrolysate that is optically inactive or practically so.

THE INTERFACIAL ADSORPTION OF GELATIN AS A FUNCTION OF THE CONCENTRATION AND pH OF ITS SOLUTIONS.

By J. M. JOHLIN.

(From the Department of Biochemistry, Vanderbilt University School of Medicine, Nashville.)

Surface tension determinations of gelatin solutions of varied concentration at 37° by the sessile bubble method show the relationship between surface tension and solution concentration to be much the same as for solutions of surface active substances of low molecular weight. In the case of gelatin solutions a long time (sometimes in excess of 24 hours) is required for a condition of equilibrium to be reached. The rate of surface adsorption varies so greatly that the relationship of equilibrium values obtained at a varied pH is quite different from the relationship of values obtained when shorter periods of time (for example, 1 hour) are allowed to elapse before the readings are taken. The data obtained emphasize the necessity of using a truly static method, and of allowing sufficient time for equilibrium, when obtaining data to show the effect of varying conditions on the surface tension of protein solutions.

The surface tension values obtained by the sessile bubble method are very much lower than any previously obtained by other methods. This fact is ascribed to the greater probability of obtaining a condition of surface equilibrium by this method than by the other methods which have been used.

LIQUID JUNCTION POTENTIALS OF CONCENTRATED NaOH SOLUTIONS.

By FRANK URBAN.

(From the Department of Biochemistry, Washington University, St. Louis.)

Liquid junction potentials of concentrated NaOH solutions have been calculated by substituting corrected ionic concentrations and mobilities in Henderson's equation. The E. M. F. of the following cells has been determined at 25°.

(1) H_2 (1 atmosphere) | NaOH (m_1) | KCl (saturated) Hg_2Cl_2 | Hg

(2) H_2 (1 atmosphere) | NaOH (m_1) | KCl (0.1) Hg_2Cl_2 | Hg

(3) Hg | Hg_2Cl_2, KCl (0.1) | KCl (saturated) Hg_2 | Hg

The following equation should hold

$$E_3 - e_3 = E_2 - e_2 - E_1 + e_1$$

where E_1, E_2, and E_3 are observed E. M. F.; e_1, e_2, and e_3 are calculated liquid junction potentials.

Good agreement was obtained.

INDUCED OXIDATIONS IN BLOOD. OXIDATION OF LACTIC TO PYRUVIC ACID BY METHYLENE BLUE.

By W. B. WENDEL and PHILIP A. SHAFFER.

(From the Laboratory of Biological Chemistry, Washington University School of Medicine, St. Louis.)

It is known that methylene blue added to various biological systems may increase the rate of oxidation and thus resemble the effect of a respiration catalyst. An example is the increase of O_2 consumption[8] and apparent inhibition of lactic acid production[9] observed by Barron and Harrop on addition of this dye to blood. We have found[10] that added *dl*-lactate is oxidized by washed oxygenated dog erythrocytes in the presence of methylene blue. This fact rendered it possible that the decreased production of lactic acid in Barron and Harrop's experiments may be due in part to oxidation of formed lactic acid. Further study shows that the lactic acid which disappears from blood or erythrocyte suspensions in the presence of methylene blue is oxidized only to pyruvic acid, and, further, that this oxidation is quantitatively coupled with the oxidation of hemoglobin (to methemoglobin) and also of other unknown substances. The following quantitative relations have been found to exist in the system—oxygenated dog erythrocytes, methylene blue, *dl*-lactate, phosphate buffer (pH 7.4), incubated 2 to 6 hours at 37°.

[8] Harrop, G. A., Jr., and Barron, E. S. G., *J. Exp. Med.*, **48**, 207 (1928).
[9] Barron, E. S. G., and Harrop, G. A., Jr., *J. Biol. Chem.*, **79**, 65 (1928).
[10] Wendel, W. B., *Proc. Soc. Exp. Biol. and Med.*, **26**, 865 (1929).

$$\text{Ratio, } \frac{\text{lactic acid oxidized } (A)}{\text{hemoglobin destroyed } (B)} = \text{constant, and}$$

$$\text{Ratio, } \frac{\text{total } O_2 \text{ consumed}}{O_2 \text{ equivalent of } A + B} = 2 \text{ or greater.}$$

A coupled reaction is evidently involved, in which the oxidation of lactic acid (and hemoglobin) is induced in the presence of the dye and hemoglobin by unknown inductors. That the oxidation of lactic acid is not mediated by a "dehydrogenase" is indicated by the fact that molecular O_2 in the absence of either dye or hemoglobin is unable to effect it. Furthermore, lactic acid is not oxidized by leucomethylene blue and molecular O_2, which mixture is known to activate oxygen. The combined action of the dye, hemoglobin, and, presumably, another oxidizable substance is necessary. A tentative scheme to account for the coupled induced oxidations has been presented.[11]

A MICRO METHOD FOR THE ESTIMATION OF CHOLESTEROL BY OXIDATION OF THE DIGITONIDE. THE EFFECT OF VARIOUS PROCEDURES FOR SAPONIFICATION UPON APPARENT CHOLESTEROL VALUES.

By RUTH OKEY.

(*From the Department of Biochemistry, The University of Rochester School of Medicine and Dentistry, Rochester, New York, and the Department of Household Science, University of California, Berkeley.*)

In working out a new micro method for the estimation of blood cholesterol by chromate-sulfuric acid oxidation of the digitonide it has become evident that ordinary saponification procedures so alter cholesterol that it is no longer quantitatively precipitated by digitonin. Cholesteryl palmitate has accordingly been synthesized, purified, analyzed and the recovery of cholesterol from the purified ester after various saponification procedures has been studied.

Ordinary saponification with strong NaOH in alcoholic solution, followed by evaporation of the alcohol has resulted with small samples, in alteration of from 10 to 30 per cent of the cholesterol so that it is not precipitable by digitonin. When the saponi-

[11] Wendel, W. B., *Proc. Soc. Exp. Biol. and Med.* (1930), in press.

fication is carried out and the alcohol removed by a current of warm nitrogen the average cholesterol precipitable by digitonin is about 94 per cent of that taken. Likewise removal of the last of the alcohol with carbon dioxide has been found to prevent to a large extent this alteration in the cholesterol. It seems possible, therefore, that the reaction may be an oxidation. Figures are given and the bearing of the findings on the efficacy of digitonin for the separation of cholesterol from mixtures of unsaponifiable material is discussed. Commercial cholesterol prepared by saponification procedures is not completely precipitable by digitonin.

THE SECRETION OF LIPIDS INTO THE INTESTINE.

By WARREN M. SPERRY AND ROBERT W. ANGEVINE.

(From the Department of Biochemistry and Pharmacology, School of Medicine and Dentistry, The University of Rochester, Rochester, New York.)

The purpose of this work was to determine whether the endogenous, fecal lipid excretion, which has been found previously in dogs, originates in the small or large intestine. Fistulæ of the small intestine were made just above the ileocecal valve in dogs, the colon being tied off and left in place. The dogs were fed the same strictly lipid-free diet used in previous work and the excretion from the fistulæ was collected in pads of gauze from which the lipids were extracted by hot alcohol and ether. At the beginning of the feeding experiments the colon was flushed out thoroughly in each case with a dilute Dakin's solution. At the end of the experiments the contents of the colon were removed at autopsy and the lipids determined.

It was found that there is an excretion of lipids from the fistulæ of the small intestine reaching from 3 to 4 times the average excretion of dogs of the same weight in their feces while normal on the same diet. At the same time only about one-fifth or less of the average normal excretion of lipids was found to have collected in the large intestine during most of the experiments.

THE RATE OF PHOSPHOLIPID METABOLISM.

By ROBERT GORDON SINCLAIR.

(*From the Department of Biochemistry and Pharmacology, School of Medicine and Dentistry, The University of Rochester, Rochester, New York.*)

It has been found that the iodine numbers of the phospholipid fatty acids in rats raised on various diets are quite characteristic of each diet and remarkably free from individual variation. Thus the iodine numbers of the phospholipid fatty acids in the entire rat and in the skeletal muscles, respectively, are: 101 and 112 for the fat-free diet, 124 and 132 for olive oil, 127 and 140 for lard, and 145 and 161 for cod liver oil. These facts have made it possible to determine the rate of metabolism or turn-over of the phospholipids in both the entire rat and in the skeletal muscles.

The procedure is to determine the rate of change in the iodine numbers of the phospholipid fatty acids when rats raised on a fat-free diet are transferred to a diet containing 40 per cent of the calories as cod liver oil.

Graphic analysis of the data shows that the replacement process proceeds rapidly during the first few days, decreases at a regular rate from day to day, and is practically complete in 2 weeks time.

The tentative conclusion is that this replacement process is the result of a continuous metabolic breakdown either of the entire phospholipid molecule or of its constituent fatty acids, with replacement at the expense of the ingested fat circulating in the blood. The choice of the phospholipid molecules to undergo this metabolism seems to be purely a matter of chance and quite independent of the degree of unsaturation. Since the rate of the replacement process and therefore the rate of phospholipid metabolism greatly exceed the usual conception of protoplasmic wear and tear, these data seem to indicate that some part, at least, of the tissue phospholipids is involved in the metabolism of fat.

A METHOD FOR THE DETERMINATION OF BLOOD CHLORIDES.

By ROBERT C. LEWIS and NEVA L. BINKLEY.

(*From the Department of Biochemistry, University of Colorado, Denver.*)

With palladious nitrate as an indicator in the titration of silver nitrate with potassium iodide as proposed by Schneider[12] for the

[12] Schneider, L., *J. Am. Chem. Soc.*, 60, 583 (1918).

estimation of silver, a method for the determination of blood chlorides has been developed. The protein in 2 cc. of blood plasma is precipitated by the addition of 15 cc. of 2 per cent sulfosalicylic acid in a 25 cc. volumetric flask, and, after the contents of the flask are diluted to the mark and allowed to stand 5 to 10 minutes the protein is removed by filtration. To 10 cc. of the filtrate in a 25 cc. volumetric flask are added 5 cc. of standard silver nitrate solution (1 cc. = 0.64 mg. of NaCl), 0.5 gm. of kaolin, and water to volume. After standing 5 minutes the mixture is filtered and the excess of silver nitrate in an aliquot portion of the filtrate (10 cc.) is titrated to the first brown color with standard potassium iodide solution (2 cc. = 1 cc. of the $AgNO_3$ solution), with 0.2 cc. of a 0.13 per cent solution of palladious nitrate in 16 per cent nitric acid as the indicator.

The accuracy of the method has been determined by the use of a known hydrochloric acid solution and the procedure has been checked with the iodimetric titration method of McLean and Van Slyke. The indicator keeps indefinitely and is extremely sensitive, producing a color which lasts at least overnight.

Preliminary work on Folin-Wu tungstic acid filtrates has shown the possibility of using the palladious nitrate indicator in direct titration of the filtrates to which a standard silver nitrate solution has been added.

THE ESTIMATION OF CYSTINE IN TISSUE AND URINE.

By M. X. SULLIVAN, W. C. HESS, and W. D. CHASE.

(From the Division of Chemistry, the Hygienic Laboratory, United States Public Health Service, Washington.)

The Sullivan cystine-cysteine reactions have been given a thorough testing in the estimation of cystine in purified proteins in comparison with other methods and in comparison with feeding experiments. The results seem highly satisfactory. The method is now being applied to tissue extracts and to urine. These applications are more difficult and have necessitated the study of interfering material, especially in the urine, and the developing of means of overcoming interferences. The evidence accumulated indicates that normal tissue and normal urine contain but little free cystine or cysteine but do contain these compounds in com-

bined form, presumably glutathione—mainly in the reduced state in tissue and in the oxidized form in the urines worked with.

THE ACID-BASE EQUILIBRIUM IN DOGS UNDER REDUCED OXYGEN TENSION.

By E. S. GUZMAN BARRON, GEORGE A. HARROP, Jr., WILLIAM A. PERLZWEIG, and H. F. PIERCE.

(*From the Chemical Division of the Medical Clinic, and the Wilmer Opthalmological Institute, The Johns Hopkins Hospital and University, Baltimore.*)

The acid-base balance of the blood and urine was studied in four normal dogs subjected to an atmosphere containing 7 to 10 per cent of oxygen for periods varying from 2 to 6 days in a specially devised cage.[13] The following blood constituents were determined: hemoglobin, red blood cells and hematocrit, O_2 content and capacity, CO_2 content, pH, protein, chlorides, phosphates, lactic acid, organic acids, and total base. In the urine, ammonia, total nitrogen, titratable acidity, creatine, and creatinine were followed in addition. During the period of anoxemia a marked polycythemia and an increase in hemoglobin were observed accompanied by a severe reduction in O_2 and CO_2 content. The total base and pH showed no significant changes. The loss of bicarbonate in the blood was partly compensated by an increase in protein, chlorides, or organic acids.

The study is being extended to splenectomized animals.

OXYGEN UTILIZATION IN THE LEGS OF NORMAL MEN.

By MARCEL FLORKIN,* H. T. EDWARDS, D. B. DILL, and L. J. HENDERSON.

(*From the Fatigue Laboratory, Morgan Hall, Harvard University, Boston.*)

In order to study the oxygen utilization in a definite part of the body, punctures of the femoral vein have been performed on men while reclining and while standing. The blood of each puncture has been analyzed with respect to the oxygen content, the oxygen capacity, and the refractive index of the plasma.

Large individual variations of coefficient of oxygen utilization in

[13] Pierce, H. F., *Am. J. Physiol.*, **85**, 399 (1928).

* Fellow of the Commission for Relief in Belgium Educational Foundation.

the reclining posture have been found, the mean value being as low as 0.21. The low value of the coefficient of oxygen utilization implies values for cardiac output in the higher range; its variability shows the variability of the rate of blood flow in the legs.

The mean value of oxygen transport changed from 4.13 volumes per cent in the reclining posture to 11 volumes per cent in the erect posture. Even when taking liberally in consideration the increase of metabolism of the legs while standing, it appears that the rate of blood flow through the legs is twice faster in the reclining posture. The value of oxygen transport in the erect posture can be reconciled with the magnitude of cardiac output only if there is a great differentiation in rate of blood flow in various parts of the body.

Though the change in refractive index is a linear function of the change in oxygen capacity, there is no correlation between these changes and the saturation of blood in the femoral vein while standing. The change in rate of blood flow reported above is the same as the change in velocity observed in other experiments by Bock, Bill, and Edwards. The mean cross sectional area is thus the same and the increase of leg volume when standing must be considered as due to lymph accumulation and not to blood stagnation.

OXYGEN TRANSPORT.

By D. B. DILL, J. H. TALBOTT, H. T. EDWARDS, and A. FÖLLING.

(*From the Fatigue Laboratory, Morgan Hall, Harvard University, Boston.*)

One of the important factors in oxygen transport is the oxygen pressure in inspired air. We have made arterial punctures during the hardest work which could be carried on in a steady state on the ergometer. On the same two individuals at sea level and after 2 weeks at 10,000 feet, the following average results were obtained.

	O_2 capacity.	O_2 intake.	Lactic acid.	O_2 pressure in alveolar air.	O_2 saturation of arterial blood.
	vols. per cent	*l. per min.*	*mg. per 100 cc.*	*mm. Hg*	*per cent*
Sea level	22.5	2.6	63	110	91
10,000 ft	22.4	2.0	65	73	87

It will be noted that the decrease in oxygen intake, which may be considered total oxygen transport capacity for this form of work, is 2 or 3 times greater than one would expect from the decrease in oxygen saturation of arterial blood. Thus, if the saturation of mixed venous blood is 40 per cent, the arteriovenous difference is 11.5 volumes per cent at sea level and 10.6 volumes per cent at 10,000 feet, a decrease of one-twelfth while total oxygen transport capacity actually decreased more than one-fifth. These results imply a decrease in cardiac output as a result of the decrease in oxygen saturation of arterial blood and are qualitatively in harmony with the experiments of Furusawa, Hill, Long, and Lupton[14] who found that a runner at sea level can increase his oxygen intake two-fifths by breathing 40 per cent oxygen instead of ordinary air.

THE DETERMINATION OF THE pH OF SERUM WITH THE QUINHYDRONE ELECTRODE.

By E. P. LAUG and D. WRIGHT WILSON.

(*From the Department of Physiological Chemistry, School of Medicine, University of Pennsylvania, Philadelphia.*)

A study of the reliability of the quinhydrone electrode for use in the pH determinations of serum and plasma was undertaken. A capillary quinhydrone electrode chamber of 0.1 cc. capacity was devised. All determinations on serum were made at 38° and checked with the hydrogen electrode. The quinhydrone electrode drifted acid 0.03 pH in 15 seconds to 0.2 pH in 1 minute, making it necessary to extrapolate all readings to zero time. Individual extrapolated readings checked among themselves within 0.02 to 0.04 pH. In thirty-four determinations on the sera of thirteen dogs, the quinhydrone electrode averaged 0.02 pH more acid than the hydrogen electrode. The maximum spread of this acid correction amounted to 0.08 pH while the maximum deviation from the average was never greater than 0.04 pH.

Severe hemorrhage was found to have no effect on the quinhydrone correction. A comparative study of serum and plasma showed the quinhydrone correction to be the same for both.

[14] Furusawa, K., Hill, A. V., Long, C. N. H., and Lupton, H., *Proc. Roy. Soc. London, Series B*, 97, 155 (1924).

The observation of Havard and Kerridge[15] that freshly drawn blood becomes 0.05 pH more acid within 6 minutes if kept at 38° has been substantiated with both hydrogen and quinhydrone electrodes.

THE INTERFEROMETRIC DETERMINATION OF ALCOHOL IN BLOOD.

By JOSEPH C. BOCK.

(From the Department of Physiological Chemistry, Marquette University Medical School, Milwaukee.)

The great majority of alcohol determinations remove the alcohol from the blood by means of vacuum distillation and estimate it by some volumetric procedure or by refractometry.

In the present method a comparatively small amount of blood (10 to 20 ml.) is treated with a phosphomolybdic acid reagent to remove the proteins. The filtrate is distilled until about 90 per cent of the original volume has been collected in the receiver. The alcohol in the distillate is determined by comparison with an alcoholic solution in a Zeiss liquid interferometer. This instrument is capable of measuring the differences in concentration of liquids within 0.0005 per cent. The amount of liquid necessary for a single determination is 10 ml., when the largest cell (40 mm.) is used.

Experiments with very dilute alcohol solution show that the alcohol was recovered within 0.005 per cent. Small amounts of alcohol added to blood which had been previously freed from volatile substances, by partial evaporation *in vacuo*, were recovered within the same limits.

Lactic acid, glycerol, fatty acids, and carbon dioxide do not interfere with the determination. We hope to overcome the occasional occurring influence of acetone by a special reagent.

[15] Havard, R. E., and Kerridge, P. T., *Biochem J.*, **23**, 600 (1929).

A MICRO COLORIMETRIC METHOD FOR THE QUANTITATIVE ESTIMATION OF IODINE IN BLOOD.

By R. G. TURNER.

(*From the Department of Medical Research, Detroit College of Medicine and Surgery, Detroit.*)

The investigation includes a method for the quantitative estimation of iodine in 10 cc. of blood with records showing the amount found in normal and pathological conditions.

The blood is ashed in an open dish by the aid of oxygen and finally extracted with absolute alcohol in the form of barium iodide. The iodine is oxidized to iodic acid and liberated in the presence of starch by addition of an excess of potassium iodide. The blue color produced is compared colorimetrically in a micro colorimeter against a 0.001 mg. standard. The color production is sensitive within 0.0001 mg. of iodine while the method on a whole is accurate within 10 to 15 per cent.

BLOOD UREA NITROGEN BY DIRECT NESSLERIZATION.

By JOSEPH M. LOONEY.

(*From the Department of Physiological Chemistry, Jefferson Medical College, Philadelphia.*)

The determination of blood urea nitrogen is one of the most difficult experiments that the student is called upon to perform.

Many attempts have been made to overcome the difficulties of the methods of distillation and aeration by direct Nesslerization, but these methods have not proved successful.

By the use of gum ghatti it is possible to protect the colloidal colored mercuric iodide compound of ammonia so that the solution remains clear even under the most adverse conditions. The gum ghatti will prevent the turbidity which invariably follows when tap water is used for dilution.

For the determination 2 drops of a 15 per cent alcoholic urease solution are added to 5 ml. of filtrate and 2 or 3 drops of buffer solution in a test-tube. The tube is then placed in a water bath at 55° for 30 minutes. Thereupon 0.15 ml. of 2 per cent gum ghatti and 1.25 ml. of Nessler's reagent are added and the volume is made up to 12.5 ml. The standard contains 0.3 mg. of nitrogen and 0.6 ml. of gum ghatti together with 5 ml. of Nessler's reagent and 8 drops of urease in 50 ml. of total volume.

THE REDUCTION OF CYSTINE IN LIQUID AMMONIA BY METALLIC SODIUM.

By VINCENT du VIGNEAUD, L. F. AUDRIETH, and H. S. LORING.

(*From the Laboratory of Physiological Chemistry, University of Illinois, Urbana.*)

A method for the preparation of cysteine has been developed based upon the reduction of cysteine in liquid ammonia by metallic sodium. A test for the presence of the sulfhydryl group in liquid ammonia has also been found depending on the production of a red color with sodium nitroprusside.

THE EXTRARENAL NEUTRALIZATION OF ACID BY AMMONIA.

By SIDNEY BLISS.

(*From the Department of Biochemistry, Tulane University Medical School, New Orleans.*)

Since it has been shown that the animal organism is able to detoxify ammonia by converting it into amide nitrogen, it follows that ammonia metabolism is reflected, in blood values, in the amide nitrogen content.

Variations in acid-base balance have been studied after giving hydrochloric acid and, in other instances, sodium bicarbonate, by stomach tube, to dogs.

In the periods following the administration of acid, there was observed a regular increase in the urinary ammonia excretion accompanied by a parallel rise in the amide nitrogen content of blood; and in the urine the ratio of total nitrogen to ammonia nitrogen decreased, showing that at the time of increased blood amide values and increased elimination of urinary ammonia there was a relative as well as an absolute increase in the amount of acid neutralized by ammonia.

When sodium bicarbonate was administered, in similar fashion, the opposite result was obtained; a decrease in urinary ammonia elimination, a fall in blood amides, and a rise in the ratio of total nitrogen to ammonia nitrogen in urine.

The values for blood amides are those for arterial blood, and show, thereby, that increases and decreases in the amount of ammonia used for acid neutralization throughout the body are reflected in the general systemic level and involve no special functional activity of the kidney.

CHEMICAL STUDIES OF TOAD POISONS.

By H. JENSEN and K. K. CHEN.

(*From the Laboratory of Pharmacology, Johns Hopkins University, Baltimore, and the Lilly Research Laboratories, Indianapolis.*)

In previous communications we reported our preliminary results on the study of Ch'an Su. Here we present more data on the same subject including those obtained from the venoms of other toads.

A. Ch'an Su, the Dried Venom of the Chinese Toad.—We were able to isolate the following compounds from Ch'an Su: (1) cholesterol, (2) epinephrine, (3) cinobufagin, (4) cinobufotoxin, (5) suberic acid. It was found that cinobufagin is different from bufagin, isolated by Abel and Macht from *Bufo marinus* (*agua*). Several derivatives of cinobufagin were prepared. Cinobufotoxin yields on hydrolysis with dilute hydrochloric acid the following compounds: (*a*) a decomposition product of cinobufagin which, so far could not be obtained in crystalline form, (*b*) arginine, isolated as a flavianate, and (*c*) suberic acid. This finding indicates a close chemical relationship of cinobufotoxin with the bufotoxin, isolated by Weiland and Alles from the European toad, *Bufo vulgaris*. By passing hydrochloric acid gas into the mother liquors, obtained in the preparation of cinobufagin, a chlorine-containing substance can be prepared which was thought by Kotake to be a derivative of cinobufagin. There is evidence, however, which makes us believe that this chlorine compound has not been derived from cinobufagin but from bufotalin; the latter has been obtained by Wieland and coworkers also from the European toad, *Bufo vulgaris*. In addition we were recently successful in obtaining from one fraction of the worked-up material of Ch'an Su a sixth crystalline body, a flavianate, which seems to be a salt of bufotenin, isolated by Handovsky from *Bufo vulgaris*. This flavianate has a marked pressor action in animals. Cinobufagin and cinobufotoxin show digitalis-like action.

The following empirical formulas are proposed for the present: cinobufagin, $C_{29}H_{38}O_7$, and cinobufotoxin, $C_{43}H_{64}O_{12}N_4$. These formulas may have to be revised as work on their chemical constitution goes on

B. Bufo marinus.—From the secretion of this toad from which

Abel and Macht 18 years ago had already isolated epinephrine and bufagin, we were able to isolate a nitrogen-containing substance which seems to be closely related to cinobufotoxin and bufotoxin chemically. It was found that this compound which we call marinobufotoxin has the same pharmacological action as cinobufotoxin. Bufagin which was also isolated was found to be identical with a specimen of bufagin given to us by Doctor Abel. Acetylbufagin was prepared.

The following empirical formulas are proposed with the same reservation as above: bufagin, $C_{28}H_{38}O_6$, and marinobufotoxin, $C_{42}H_{64}O_{11}N_4$.

C. Bufo arenarum.—From the secretion of *Bufo arenarum* we obtained a substance which seems to be closely related to bufagin, bufotalin, and cinobufagin chemically. Arginine was isolated as a flavianate. We also obtained indications of the presence of a nitrogen-containing substance similar to bufotoxin. As soon as more material is available this point will be settled.

THE USE OF HEAVY METAL SALTS FOR THE PREPARATION OF BLOOD FILTRATES FOR ANALYSIS.

By MICHAEL SOMOGYI.

(*From the Laboratory of the Jewish Hospital, St. Louis.*)

Heavy metals have been used for the precipitation of blood proteins mainly in the form of their hydroxides. Hagedorn and Jensen employ zinc hydroxide coupled with heat coagulation, and Michaelis and Rona introduced colloidal iron hydroxide. Precipitation with mercury was severally carried out by addition of its salts to laked blood and subsequent neutralization of the mixture. The writer applied this principle to procedures with the salts of zinc, copper, and iron besides mercury. The procedure in general is as follows: Blood is laked in water, the solution of the metal salt (sulfate) is added, and the mixture approximately neutralized with sodium hydroxide. For each metal we established the amount adequate in relation to blood proteins. The final hydrogen ion concentrations were so regulated as to not only secure the complete precipitation of proteins but also to remove as much as possible of the substances which reduce alkaline copper solutions and thus interfere with the determination of blood sugar. The

procedure works very smoothly with zinc and copper; precipitation with iron and mercury yields filtrates requiring special treatment prior to the determination of sugar.

Single blood samples were deproteinized by each of these procedures and the filtrates analyzed for (1) non-sugar reducing substances, (2) fermentable sugar, and (3) non-protein nitrogen. It was found that filtrates prepared with zinc, copper, and iron are substantially free of reducing non-sugars but mercury filtrates do contain 3 to 5 mg. per cent, in terms of glucose. As to the fermentable (true) sugar, this is quantitatively present in zinc filtrates. Added glucose is recoverable with great accuracy. If copper is used as protein precipitant, some of the fermentable sugar is in most cases carried down with the precipitate, the loss being sometimes considerable. Iron filtrates yield sugar values fairly close to true sugar, but not so consistently as in zinc filtrates. Mercury filtrates yield results mostly a few mg. per cent too high. The non-protein nitrogen is in all of these blood filtrates lower than in the tungstate filtrates of the Folin-Wu method. Zinc filtrates and copper filtrates contain identical amounts of nitrogen, on an average 13 mg. per cent lower than tungstate filtrates. The nitrogen content of iron filtrates lies between those of zinc and tungstate filtrates. Mercury filtrates contain but slight quantities of nitrogen, ranging from a trace to 10 mg. per cent. These differences suggest a possible system for the fractionation of the non-protein nitrogenous constituents of blood.

EVIDENCE OF THE ACTIVATION OF FRUCTOSE IN THE ENZYMATIC HYDROLYSIS OF SUCROSE.

By ETHEL RONZONI.

(From the Departments of Biochemistry and Internal Medicine, Washington University School of Medicine, St. Louis.)

It has been reported previously that yeast ferments equal concentrations of glucose and sucrose at the same rate. Fructose, however, ferments at a slower rate than either. A solution of the same total carbohydrate concentration but of equal parts of glucose and fructose, ferments at an intermediate rate between that of pure glucose and that of pure fructose. Sucrose hydrolyzed by acid or by sucrase separated from yeast, and then allowed to

stand overnight, ferments at the same intermediate rate. At about the optimum pH (5.5) for both sucrase activity and glucose fermentation, all sucrose present in dilute solutions is promptly inverted (3 to 5 minutes) and fermentation takes place at the rate characteristic of *glucose.* At a pH that inhibits sucrase in yeast suspensions (less than 2) *no* fermentation takes place. Therefore, the yeast used (Baker's yeast) ferments sucrose only after inversion by sucrase, and it ferments the fructose *newly split* from sucrose at the glucose rate.

The finding of Willstätter that at a pH greatly inhibiting sucrase activity, fermentation of sucrose still takes place at a normal rate, does not force us to his conclusion that sucrose is, therefore, fermented without being hydrolyzed, because we find that at any pH at which fermentation will take place at all, invert sugar is present in the solution, sucrose in the *presence* of *yeast cells* is still active; invert sugar is present, and slow fermentation takes place. At a pH low enough to prevent the formation of invert sugar in a yeast suspension, fermentation is inhibited, even glucose being unfermented.

ACTIVE GLUCOSE AT BIOLOGICAL HYDROGEN ION CONCENTRATIONS.

By JOHN M. ORT.

(*From the Division of Physics and Biophysical Research, The Mayo Foundation, Rochester, Minnesota.*)

This paper is a report of further study on the first steps of sugar oxidation by mild oxidants such as dissolved air or dilute hydrogen peroxide. By the use of oxidation potential technique it has been previously shown that at pH 10 there is present in glucose solutions a minute amount of a very active reductant which is in equilibrium with the main bulk of the comparatively inactive ordinary glucose. This "active glucose" is instantly oxidized by even as weak an oxidant as the air dissolved in ordinary distilled water. These general facts are now found to be true also for pH at or near the neutral point, although of course, the reduction intensity developed at the lower alkalinities is not so great nor is the oxidation of the active reductant so fast.

SUGAR OF NORMAL URINE.

By MARK R. EVERETT and FAY SHEPPARD.

(From the Department of Biochemistry and Pharmacology, University of Oklahoma Medical School, Oklahoma City.)

The major portion of this material is readily soluble in methyl alcohol, less soluble in acetone, and insoluble in ether. It is not destroyed by rapid evaporation of slightly acid urine at 60°, under reduced pressure. It is not precipitated by mercuric or silver salts from slightly acid urine, nor by barium salts from neutral urine. Night urine generally shows no loss of reducing action as a result of bromination at room temperature for 48 hours. The brominated urine often contains more reducing material than the original urine, because of the action of the hydrobromic acid upon the hydrolyzable sugar. Bial's test, Saliwanoff's test, etc. become clearly positive in the brominated, hydrolyzed urine, because bromination removes the interfering phenols. Since bromination excludes the presence of large amounts of known aldoses or aldehyde acids, while 2-furaldehyde can be produced from normal urine, it is probable that the major portion of the reducing material is a ketose, perhaps a pentose or methyl pentose. There is a smaller portion of the free sugar which may not be carbohydrate in nature, since it resists the action of potassium hydroxide solution at 100°.[16]

Before hydrolysis, the hydrolyzable sugar is usually not brominated; after hydrolysis, the result depends upon the analytical method employed.

THE RATE OF DIALYSIS OF NORMAL AND DIABETIC BLOOD SUGAR OF HUMAN SUBJECTS.

By MARION BELL and ISRAEL S. KLEINER.

(From the Department of Physiological Chemistry of the New York Homeopathic Medical College and Flower Hospital, New York.)

Former experiments by one of us[17] upon the rate of dialysis of blood of diabetic dogs showed a delay or complete interruption of the dialysis during one or more periods. Normal dog blood to

[16] Everett, M. R., Shoemaker, H. A., and Sheppard, F., *J. Biol. Chem.*, **74**, 739 (1927).

[17] Kleiner, I. S., *J. Biol. Chem.*, **34**, 471 (1918).

which glucose had been added dialyzed regularly, without delay or interruption. This difference suggested the existence of "combined" sugar in diabetic blood.

Inasmuch as this earlier work was done entirely upon dogs, it seemed necessary to determine whether the phenomenon could be observed in human blood. The present work is an extension of experiments reported briefly before The Thirteenth International Physiological Congress.[18]

In the present series of experiments human diabetic blood was dialyzed against Ringer's solution and samples taken for analysis every 15 minutes. These results were compared with dialyses of (1) normal human blood with added glucose, (2) normal human blood without glucose, and (3) solutions of pure glucose in Ringer's solution.

It was found that the glucose solutions and the normal bloods without added sugar dialyzed in a regular, gradual manner, giving a practically straight curve. The diabetic blood sugar dialyzed very irregularly, with frequent delays in the fall and even rises of the blood sugar values. Normal human blood to which glucose had been added also dialyzed very unevenly as compared with the extremely regular curves mentioned above. The dialysis was, however, slightly faster and a trifle less irregular than that of diabetic blood.

THE GLUCOSE TOLERANCE CURVES IN PHLORHIZINIZED DOGS WITH AND WITHOUT GLUCOSE.

By HARRY J. DEUEL, Jr.

(*From the Department of Biochemistry, University of Southern California School of Medicine, Los Angeles.*)

The cause of the abnormally high glucose tolerance curves obtained with phlorhizinized dogs after the feeding of standardized amounts of glucose, has been investigated in order to determine whether the glycosuric action of phlorhizin is primarily of renal origin or whether a true diabetes occurs. The diabetic symptoms occurring concomitantly in fasted phlorhizinized animals, such as the acidosis, the high protein metabolism, and the decreased

[18] Kleiner, I. S., and Bell, M., *Am. J. Physiol.*, 90, 410 (1929).

glucose tolerance have been ascribed[19] a secondary action of this drug resulting from carbohydrate depletion of the tissues.

The glucose tolerance curves following the ingestion of 16 gm. of glucose by phlorhizinized dogs have been compared during acidosis with those following the abolition of acidosis by previous carbohydrate feeding. Two experiments in which this amount of glucose was administered twice at 3 hour intervals to previously fasting phlorhizinized dogs indicate that the second test, in which no ketosis was present, simulated the normal curve much more than that obtained after the first administration of glucose at which time ketosis existed.

In a second series of experiments on two phlorhizinized animals, glucose solution was introduced by stomach tube at a constant rate for 12 hours. After the abolition of the ketosis (6 to 8 hours), when the blood sugar level had become stabilized at a higher level than normal, an additional 16 gm. of glucose were given by stomach tube and the tolerance curve followed. In both cases these values increased much less than occurred in the same animals during ketosis.

Thirdly, two experiments were made on a single phlorhizinized dog in which the acidosis had been completely abolished by the previous administration of 250 and 200 gm. of sucrose on successive nights. The blood sugar rose only a minimal amount and returned to the preglucose level within 2 hours when 16 gm. of glucose were administered on each of the following mornings. The results were directly comparable to those obtained on normal animals after heavy carbohydrate feeding. These experiments all indicate that the diabetic glucose tolerance curves obtained on previously fasted phlorhizinized dogs are not the result of an intrinsic impairment in the ability to oxidize carbohydrate depletion such as ketosis, or decreased insulin production.

[19] Deuel, H. J., Jr., Wilson, H. E. C., and Milhorat, A. T., *J. Biol. Chem.*, **74**, 265 (1927).

THE ACTION OF EPINEPHRINE AND INSULIN UNDER ANAEROBIC CONDITIONS.

By CARL F. CORI AND K. W. BUCHWALD.

(*From the State Institute for the Study of Malignant Disease, Buffalo.*)

Frogs were kept in an atmosphere of nitrogen at a temperature of 15°. Traces of oxygen were removed by allowing the gas to pass over copper heated in a silica tube. After 3 hours the animals were covered with CO_2 snow until frozen solid and cut into thin slices. The tissue was submerged in ice-cold 2 per cent H_2SO_4 and after some time was passed through a meat grinder. The tissue by now finely minced was washed several times on a Buchner funnel and the extract was precipitated with $HgSO_4$ as described by West, Scharles, and Peterson. Sugar and lactic acid were determined, the latter after treatment of the filtrate with $CuSO_4$ and $Ca(OH)_2$. Anaerobiosis produces an increase in glucose and lactic acid; the former has its origin in liver and the latter in muscle glycogen. When epinephrine is injected at the beginning of anaerobiosis, hydrolysis of liver and muscle glycogen is accelerated. An injection of insulin 18 to 42 hours previous to the experiment inhibits the hydrolysis of liver glycogen which takes place under anaerobic conditions but is without influence on lactic acid formation. The accelerating effect of epinephrine on hydrolysis of liver and muscle glycogen is inhibited by insulin under aerobic as well as anaerobic conditions. These results are explained by the assumption that insulin and epinephrine have an opposite effect on the enzyme systems in liver and muscle.

THE SUPRARENAL EPINEPHRINE-LIPOID COMBINATION.

By ALFRED E. KOEHLER AND L. EICHELBERGER.

(*From the Department of Medicine, University of Chicago, Chicago.*)

We have previously ascribed certain metabolic effects to the suprarenal lipoid fraction obtained by alcohol and benzene extraction. Anaerobic autolytic or mild acid hydrolysis produced a water-soluble fraction of increased calorigenic activity but these processes also decreased the stability of this preparation. It was customary to wash the benzene lipoid solutions until the wash water gave none of the epinephrine color reactions. After the

washing the lipoid was further tested for epinephrine after 10 per cent acetic acid hydrolysis for 1 hour and usually none was found. It was, however, observed that the lipoid washed free from epinephrine with water or dilute cold acetic acid would turn a dark cherry-red if exposed in a thin film to the air. Hydrolysis with 5 or 10 per cent acetic acid then showed that relatively large amounts of epinephrine or an epinephrine-like substance could be obtained. The water hydrolysate gave the characteristic epinephrine color and pressor reactions. The lipoid of the cortex separated from the medulla as completely as possible by dissection also gave a definite amount of epinephrine by this method.

Hydrolysis of the lipoids with dilute acetic acid at 100°, in contact with air, however, gives an optimum yield in from 10 to 30 minutes following which the epinephrine again disappears and after an hour of hydrolysis practically none remains. Acid hydrolysis in nitrogen or digestion with a highly purified lipase preparation (which also contained small amounts of trypsin) under anaerobic conditions greatly increased the yield of epinephrine from the lipoids. The lipoid fraction seems to have a specific destructive action on epinephrine not encountered when pure epinephrine is heated for similar periods of time with dilute acids.

The water-soluble fraction obtained from the suprarenal cortex lipoid by acid hydrolysis, by lipase digestion, or prolonged autolysis in which epinephrine has been demonstrated also contains the orally effective calorigenic substance. The relation of the latter substance to epinephrine, epinephrine-like substances, and to a possible precursor of epinephrine are now being studied.

FURTHER OBSERVATIONS ON THE EFFECT OF INORGANIC ELEMENTS IN NUTRITIONAL ANEMIA.

By HOWARD H. BEARD and VICTOR C. MYERS.

(*From the Department of Biochemistry, School of Medicine, Western Reserve University, Cleveland.*)

In a previous preliminary report[20] we have shown that young rats made anemic by 6 weeks feeding on whole milk could be cured by the addition of 0.5 mg. of Fe, daily, as $FeCl_3$, in 6 weeks. When

[20] Myers, V. C., and Beard, H. H., *J. Am. Med. Assn.*, **93**, 1210 (1929).

traces of Cu, Ni, Ge, As, or Mn, were fed in addition to 0.5 mg. of Fe daily, recovery of hemoglobin took place in 2 to 3 weeks. Since the H_2S filtrate from our Fe solutions brought about recovery in the same length of time as our original Fe solution, it would not seem that the results with this element could be due to the contamination of any element precipitable by H_2S that might have been present as an impurity in the Fe.

We wish to present further data at this time to show that several other elements have essentially the same Fe supplementing action in nutritional anemia as those mentioned above. Ti, Zn, Rb, V, Cr, Se, and Hg, all brought about recovery in practically the same time as the other elements, namely about 2 to 3 weeks, when proper doses were employed.

The action of several elements upon adult animals was also studied. In confirmation of the work of Waltner[21] it was found that when 1 per cent Co was added to our stock diet, the hemoglobin was raised to 18 to 20 gm. per 100 cc. from the normal of about 13.5 gm. and erythrocyte count to 12 to 13 millions per c.mm. from the normal of about 8.5 millions. V when added to the stock diet to the extent of 1 per cent produced much the same effect. Ge, in 0.1 mg. daily doses in milk, likewise gave high values for both Hb and red blood cells.

Zn, Cu, Fe, Mn, Ni, and As, fed in the same doses in milk as used in the anemia experiments, all gave values of Hb from 16 to 19 gm. per 100 cc., but with only slight rises in the red blood cell counts. When these supplements were continued, all values returned to normal in 2 to 3 weeks, except in the cases of Co and V, where the animals died.

THE COPPER CONTENT OF INFANT LIVERS.

By D. B. MORRISON and THOMAS P. NASH, Jr.

(*From the Department of Chemistry, School of Biological Sciences, University of Tennessee, Memphis.*)

Analyses of twenty-five infant livers, ranging in age from birth to 2 years, show an average copper content of 24.0 mg. per 1000 gm. of fresh tissue. The lowest value was 6.9 mg. and the highest 57.6 mg. Seven adult livers gave an average of 4.0 mg. of copper,

[21] Waltner, K., *Arch. exp. Path. u. Pharmakol.*, **141**, 123 (1929).

with the lowest 1.6 and the highest 8.5 mg. The results indicate a possible storage of copper in the liver of the infant. These values are comparable to those reported in the literature.

For the analyses made in this laboratory the method of Biazzo as modified by Elvehjem and Lindow was used. All reagents, including the distilled water, were tested and found to be free from copper.

CRYSTALLINE GLUTATHIONE.

By EDWARD C. KENDALL, HAROLD L. MASON, and BERNARD F. McKENZIE.

(From the Section on Chemistry, The Mayo Foundation, Rochester, Minnesota.)

The isolation of glutathione from yeast by an adaptation of Hopkins' method and the use of cold water and benzene will be described. The chemical properties of glutathione together with its digestion by enzymes and its decomposition in cold water will be given. A method for the determination of glutathione in tissue extracts together with the amounts of glutathione present in various tissues will be described and a comparison with amounts previously determined by other methods will be given. The new method based on the reduction of ferricyanide by glutathione in a narrow range of hydrogen ion concentration together with the determination of a blank in the presence of formaldehyde which prevents the oxidation of the SH-group, furnishes an accurate method for the estimation of glutathione. A study of the chemical properties and decomposition products of glutathione has shown conclusively that it is glutamyl cysteinyl glycine. The free amine group of the glutamic acid is gamma to the carboxyl group which is attached to the amine group of cysteine.

THE INFLUENCE OF YEAST ON NITROGEN RETENTION IN NORMAL AND DEPANCREATIZED DOGS.

By E. S. NASSET and H. B. PIERCE.

(From the Department of Vital Economics, The University of Rochester, Rochester, New York.)

One normal dog and four completely depancreatized dogs were fed a basal diet with additions of bakers' and starch-free yeast in 4 to 6 day periods. The control dog showed a greater nitrogen

retention in the yeast periods than in the control periods, amounting, in the first weeks of the experiment, to 150 per cent of the extra nitrogen ingested in the yeast. The loss of nitrogen in the depancreatized dogs was less in the yeast periods than in the control periods, with no significant alteration in the D:N. There was no apparent correlation between the partition of waste nitrogen to the urine and feces and the addition of yeast to the diet.

FURTHER STUDIES ON THE BIOCHEMISTRY OF AVITAMINOSIS.

By BARNETT SURE, M. C. KIK, AND MARGARET ELIZABETH SMITH.

(*From the Departments of Agricultural Chemistry and Home Economics, University of Arkansas, Fayetteville.*)

Vitamin A Deficiency.—Further studies do not establish any anemia in this avitaminosis. There are no demonstrable changes in the liver glycogen, apparent and true sugar. Five animals out of twelve showed evidence of acidosis, three mild, and two marked. The two cases of marked acidosis were associated with pneumonia.

Vitamin B Deficiency.—There is a marked reduction in the liver glycogen during advanced stages of polyneuritis after inanition has set in.

Vitamin D Deficiency.—There is a suggestion of an anemia, characterized mainly by a lack of erythropoiesis. Some animals show reduction in the concentration of hemoglobin associated with reduction of concentration of phosphorus in the serum.

Vitamin G Deficiency.—A number of animals show secondary anemia during periods uncomplicated by inanition. There are no significant changes in the concentration of true and apparent blood sugar. We are unable to produce the dermatitis during the winter on the same diet which produced the characteristic skin lesions during the previous summer.

DESTRUCTION OF VITAMIN A BY RADIOACTIVE MATERIALS.

By ALBERT G. HOGAN, CHARLES L. SHREWSBURY, AND GERALD F. BRECKENRIDGE.

(*From the Departments of Animal Husbandry and of Chemistry, University of Missouri, Columbia.*)

One of us (G. F. B.) had obtained a preparation of radiothorium and so the opportunity presented itself for studying the lability

of vitamins when exposed to these unique substances. Radiothorium was loosely cemented to a small container and covered with mica to prevent loss of the active material. The α-particles are unable to penetrate mica, and the intensity of the β-rays is somewhat reduced. The γ-rays are not appreciably affected. The activity of the preparation, measured by the γ-ray method, was equal to 25 mg. of radium.

The stability of vitamin A was first studied, and milk fat was chiefly used as a source of material. The radiothorium was suspended directly over the fat, at a distance of approximately 2.5 cm. for a period of 4 days. On the 3rd day the area of fat directly beneath the radiothorium was distinctly bleached, and by the end of the 4th day it was almost entirely colorless. The entire sample was then transferred to a glass container and stored in the refrigerator. When removed several days later for use, it was found that the bleaching process had continued until the sample was colorless. This material no longer gave any evidence of vitamin A activity. It would not support growth of rats nor would it cure or prevent ophthalmia.

PRODUCTION OF STEROLS BY VARIOUS MOLDS.

By L. M. PRUESS.

(From the Departments of Agricultural Chemistry and Agricultural Bacteriology, University of Wisconsin, Madison.)

In a preliminary survey, 56 molds were used to inoculate a synthetic, inorganic medium containing glucose as the source of carbon. Of these molds twenty-seven showed good growth. The autoclaved, dried, and finely ground pads of sixteen of the twenty-seven molds were irradiated with ultra-violet light and fed to rachitic rats to test their vitamin D potency. Five kinds of mushrooms, gathered in the open, were also tested biologically for their antirachitic activatability.

Eight molds, representing the principal species, which grew well on the synthetic medium and gave good results in the animal experiments were grown in quantity and analyzed for sterol by the gravimetric digitonide method. The dried mold pads were extracted 6 hours with absolute alcohol. Free sterols in this extract were precipitated directly, and total sterols were deter-

mined after saponification with 1 and 10 per cent alcoholic potassium hydroxide. The total alcohol-soluble sterols varied from 0.1 to 1.0 per cent of the dry weight of the fungus material, and 95 per cent of these sterols were present in the free state. The residue from the alcohol extraction was refluxed with 10 per cent alcoholic potassium hydroxide to give an additional quantity of sterol which ranged from 0.04 to 0.40 per cent of the dry pad. The amount of sterol present in the fungi studied was found to be dependent upon the species, strain, age of culture, and composition of the medium.

UREA CONCENTRATIONS IN THE BLOOD OF RATS AS AFFECTED BY SUCKLING THE YOUNG.

By HELEN T. PARSONS.

(From the Department of Home Economics, University of Wisconsin, Madison.)

Remarkably high concentrations of urea were regularly observed in the blood of lactating rats on high casein rations following unilateral nephrectomy during the course of a study[22] on the physiological response of rats to diets rich in protein, in the Laboratory of Physiological Chemistry, Yale University.

It has been shown that the increased food intake of the lactating females accounts only in part for the extreme values observed. Even during fasting prompt and striking increases in the concentration of urea (as great as 42 mg. per 100 cc. of blood, in 70 minutes) in the blood of mother rats, and to a less degree, of hares and guinea pigs, have been demonstrated to follow the act of suckling the young. The magnitude of the increase is not closely correlated with the amount of milk nursed. An occasional slight increase results from the taking of the blood sample. No increases in the urea content of the blood of cows have been shown to result from the withdrawal of milk from the udder by manual expression, milking machine, or the nursing of the calf.

[22] Parsons, H. T., Smith, A. H., Moise, T. S., and Mendel, L. B., *Arch. Path.*, in press. Preliminary report, *Proc. Soc. Exp. Biol. and Med.*, **25**, 681 (1928).

THE FUNDAMENTAL FOOD REQUIREMENTS FOR THE GROWTH OF THE RAT.

VI. THE INFLUENCE OF THE FOOD CONSUMPTION AND THE EFFICIENCY QUOTIENT OF THE ANIMAL.

By LEROY S. PALMER and CORNELIA KENNEDY.

(From the Section on Animal Nutrition, Division of Agricultural Biochemistry, University of Minnesota, St. Paul.)

We began a study of this problem early in 1927, and have employed several hundred animals in various phases of its progress. We first found that appreciable differences in the average growth curve, uniformly apparent when different diets are fed *ad libitum*, practically disappear when the food intake is equalized, provided the adjustment is on the basis of digestible dry matter. Very large individual variations from the average were found to be due to great differences in their efficiency quotient for food utilization (gain per gm. of food per 100 gm. of body weight). By using animals with the same predetermined efficiency quotient and equalizing the food intake, no difference could be detected between yeast and wheat embryo extract as sources of vitamins B and G for growth. The so called stimulating effects of fresh lettuce, liver, yeast, and carrots, in various combinations, on growth appear to be due merely to effects on the appetite, when studied by this method. There is a marked sex difference in rats in their efficiency quotient which appears to be a major factor in determining growth rate. Individual variation in efficiency quotient, likewise, is a major, if not the controlling cause of individual variation in gain in weight of animals on the same diet.

CHANGES IN METABOLIC RATE, FUEL, AND WATER BALANCE ON WITHDRAWAL OF DOGS FROM MORPHINE.

By H. G. BARBOUR, D. E. GREGG, and L. G. HUNTER.

(From the Department of Physiology and Pharmacology, University of Louisville, Louisville, and the Department of Vital Economics, School of Medicine and Dentistry, The University of Rochester, Rochester, New York.)

Three dogs after about 2 months on morphine exhibited high basal metabolism (respiration chamber). Two fat-fed dogs showed over 40 calories per square meter per hour; a casein-fed

dog 55. (A 6 months addicted, better nourished, fat-fed dog had a normal rate.) In the two Cowgill plan synthetic diets used, casein and lard respectively afforded 75 per cent of the total calories.

Morphine withdrawal (on strictly constant food intake) reduced all high metabolic rates; readdiction increased them. Morphine, therefore, early in addiction, proved to be a *metabolic stimulant.*

On withdrawal, *basal·protein* combustion was reduced 35 to 65 per cent notwithstanding a much increased *total* nitrogen output. Carbohydrate combustion increased 4- to 6-fold in all but the casein-fed dog. Fat combustion decreased in the two fat-fed short addiction dogs, from 80 per cent to 60 per cent of the basal metabolism. Serum lipids were reduced. Fat absorption was practically complete, as during addiction.

As we showed before, such dogs lose excess liver and muscle fat on withdrawal; this fat must, therefore, return to the depots.

During withdrawal water intake, urine volume, and their difference are all high. The weight curves indicate storage of water, especially at first. Water and carbohydrate changes are probably related.

FURTHER STUDIES ON CRYSTALLINE INSULIN.

By H. JENSEN and A. DeLAWDER.

(*From the Laboratory of Pharmacology, the Johns Hopkins University, Baltimore.*)

A. Adsorption of Insulin on Charcoal.

The adsorption of insulin on charcoal was studied with the purpose of seeing whether it would be possible to obtain an insulin preparation which was more active than crystalline insulin which contains about 24 international units per mg. Dingemanse claims to have been successful in preparing an insulin product which contained about 150 international units per mg. We were very fortunate in obtaining a sample of charcoal and of insulin (organon) from Dr. Dingemanse which according to her should yield these highly active insulin preparations. Starting from crystalline insulin and submitting it to the adsorption on charcoal (preparation of Dr. Dingemanse) under the conditions given by

Dingemanse and comparing this product with the crystalline insulin started from, we were not able to secure a preparation more active than crystalline insulin.

Repeating the work of Dingemanse as closely as possible, using the same insulin (organon) and charcoal as employed by Dingemanse, we were not able to obtain a preparation with a higher activity than crystalline insulin.

With insulin, prepared exclusively from pig pancreas, we likewise obtained only negative results.

We therefore cannot substantiate the findings of Dingemanse of an insulin preparation more active than crystalline insulin.

B. Can Insulin Be Activated?

In connection with the work on the adsorption of insulin on charcoal we were interested to see if it would be possible to activate insulin by certain activators as has been claimed by some investigators. We have tried to activate crystalline insulin as well as commercial amorphous insulin preparations. So far we have not been able to find any indication that it is possible to activate insulin by the addition of activators. As activators we employed the following: (*a*) enterokinase, (*b*) blood serum, and (*c*) yeast juice.

NUTRITION STUDIES WITH COTTON SEED DEMONSTRATING THE TOXICITY OF GOSSYPOL TO THE RAT.

By E. M. NELSON and D. BREESE JONES.

(*From the Bureau of Chemistry and Soils, United States Department of Agriculture, Washington.*)

Various levels of gossypol have been fed to rats receiving an adequate diet. C.05 per cent of gossypol in the diet caused but a slight diminution of the growth rate and no other untoward effects. 0.4 per cent of gossypol resulted in death of the animals in 6 to 9 days. The animals responded with marked uniformity to intermediate levels. Cottonseed products which had been examined chemically for free gossypol produced retardation of the growth rate in proportion to the free gossypol present in similar feeding experiments.

THE SEX VARIATIONS OF THE LIVERS AND THE LIVER OIL OF THE NORWEGIAN COD.

By PHILIP B. HAWK.

(From the Food Research Laboratories, Inc., New York, and the Research Laboratories of Scott and Bowne, Bloomfield, New Jersey.)

This investigation embraced the study of 1779 cod fish caught during the height of the cod fishing season (March 1 to 15) in the Lofoten area of Norway as the fish were preparing to spawn. Of these fish 59 per cent (1041) were females and 41 per cent (738) were males. The female fish as a class were larger than the males and their livers were larger and of a lighter color. The 1779 fish yielded a total of 420 liters of liver of which 300 liters (71 per cent) were from the female cod and 120 liters (29 per cent) were from the male cod. There were 615 livers per hektoliter of male livers and 347 livers per hektoliter of female livers. The yield of crude cod liver oil as produced by a modified "direct steam" process was 43.3 per cent for the male livers and 37.1 per cent for the livers of the females.

It is worthy of note that the oil produced from the livers of both the female and male cod of the Lofoten area of Norway, just previous to spawning, possesses about the same general qualities, but that the amount of oil in the female liver is somewhat less per unit of liver substance than is present in the male liver, although the female livers are about 80 per cent larger and 40 per cent more numerous than the male livers. This might indicate that the female cod in the formation of roe in preparation for spawning draw upon their liver reserves to such an extent that the store of liver oil is depleted. However, in spite of the possible lessening of the percentage of oil, the property of the oil from the livers of the female cod which is of outstanding importance, namely its vitamin potency, is not appreciably different from that of the liver oil of the male cod.

It may possibly be of interest to record the fact that the oil from the livers of the male cod gave slightly better values in the vitamin assays, tintometer examinations, and free fatty acid determinations than did the oil from the livers of the female cod. Nevertheless, when each of these observations is considered apart from the others the variation is without significance. The oil from the livers of the two sexes showed no variation as to color, odor, or taste.

THE NATURE OF THE MATERIAL EFFECTIVE IN PERNICIOUS ANEMIA. IV.*

By EDWIN J. COHN, THOMAS L. McMEEKIN,† AND GEORGE R. MINOT.

(*From the Department of Physical Chemistry in the Laboratories of Physiology, Harvard Medical School, and the Thorndike Memorial Laboratory, Boston City Hospital, Boston.*)

It is just 3 years since the extraction from liver of the active principle effective in pernicious anemia was reported to this Society at its Twenty-first Annual Meeting. At that time the chemical dissection of the liver, undertaken in order to discover the nature of the substance concerned with blood regeneration in this condition, had effectively concentrated this constituent in certain fractions which "besides being freed from lecithin and ordinary lipoids, and all but a trace of protein" had "been freed from all but a trace of sulfur and iron."[23] The process of concentrating the active principle by removing one class of substances after another, as they proved inert, has since invariably been followed.

The purest extract that had been prepared at that time, when treated with basic lead acetate yielded an active carbohydrate-free filtrate, the active constituent of which was precipitable by phosphotungstic acid.[24,25] Since the active principle was neither protein, carbohydrate, nor lipoid, and was precipitated by phosphotungstic acid or by mercuric acetate, it appeared to be a nitrogenous base or polypeptide. Further fractionation yielded an active concentrate from which proteoses, peptones, and polypeptides had been removed, and suggested that the active principle was rather a base than a peptide.[26]

* This investigation has been aided in part by grants from the Farnsworth and the De Lamar Mobile Research Fund of the Harvard Medical School.

† Fellow of the Leopold Schepp Foundation.

[23] Cohn, E. J., Minot, G. R., Fulton, J. F., Ulrichs, H. F., Sargent, F. C., Weare, J. H., and Murphy, W. P., *J. Biol. Chem.*, **74**, p. lxix (1927).

[24] Cohn, E. J., Minot, G. R., Alles, G. A., and Salter, W. T., *J. Biol. Chem.*, **77**, 325 (1928).

[25] West, R., and Nichols, E. G., *J. Am. Med. Assn.*, **91**, 867 (1928).

[26] Cohn, E. J., McMeekin, T. L., and Minot, G. R., *Am. J. Physiol.*, **90**, 316 (1929).

Although fractionation has proceeded by the elimination of those substances whose presence was indicated by their precipitability by heavy metals, by color tests, or by some comparable reagent, the procedures that have been evolved have for the most part depended upon the use of organic solvents and precipitants, "since in highly purified solutions heavy metals appear to destroy activity."[26] The slight solubility of the active principle in 95 per cent alcohol has permitted of its extraction in this solvent from sufficiently purified concentrates, leaving undissolved those substances which gave the biuret test to earlier extracts. Upon concentration of this alcoholic solution the active principle was, in large part, precipitated leaving in solution all but a trace of the blood pressure-reducing substances. Freed thus of those constituents which give specific protein reactions, and of those which reduce blood pressure, the active principle could be injected intravenously. This first extract to be injected intravenously "I.E." was the starting material for all subsequent fractionation, and rendered possible subsequent testing by intravenous injection.

The studies that led to the preparation of fraction "I.E." demonstrated that, although the active principle could be precipitated by means of alcohol from sufficiently concentrated aqueous solutions, it had a definite solubility in alcohol-water mixtures. The extract "I.E." readily dissolved in 90 per cent alcohol from which a smaller fraction, containing the active principle, was precipitated by addition of an equal volume of ether. The tryptophane and tyrosine present passed into the filtrate in this separation. Next, the active principle was extracted from the precipitate by a mixture consisting of 1 volume of water, 9 of alcohol, and 4 of ether, whereas a large number, but not all, of the substances giving the diazo test remained in the precipitate. Certain phosphorus-containing substances present were removed by precipitating the active principle from a mixture containing 1 volume of water, 12 of alcohol, and 6 of ether. The phosphorus-containing substances passed into the filtrate.

The active precipitate gave no biuret test, nor Molisch, Pettenkoffer, Millon's, nitroprusside, or diacetyl reaction. Besides being free from proteins, carbohydrates, and lipoids, it appeared to be free of tryptophane and tyrosine, arginine, cystine, and creatinine, phosphorus, iron, and sulfur. It gave no precipitate

with trichloroacetic, picric, or picrolonic acids or with flavianic acid in acid solution. It contained substances which precipitated not only with phosphotungstic acid and mercuric sulfate in acid solution but with gold chloride, platinic chloride, and silver nitrate. Whereas the activity had been increased 4-fold, the blood pressure-reducing substances had been diminished 40-fold in concentration as compared with "I.E." A single injection of 2 gm. sufficed to give a maximal reticulocyte response.

When this product was dissolved in a small volume of 20 per cent alcohol and placed in the cold a white precipitate, crystalline in nature, slowly appeared. This material was redissolved and reprecipitated from 20 per cent alcohol containing 5 per cent ether, thus freeing it, in large part, from other constituents. When 0.95 gm. was intravenously injected a satisfactory reticulocyte and erythrocyte response ensued.

The material injected still contained substances which were precipitated by mercuric sulfate from solution in sulfuric acid. Upon repeated recrystallization, these were removed, and a crystalline substance obtained, all of the nitrogen of which was α-amino nitrogen. Further the formol titration demonstrated the presence of carboxyl groups equal in number to the amino groups, and the nitrogen content and solubility of the substance suggested leucine. 1 gm. of the highly purified crystals proved inert.

The substance or substances which had separated with these crystals in the cold were, in large part, precipitable by mercuric sulfate from sulfuric acid solution. The effort has repeatedly been made[24,26] to use this reagent which has always given a precipitate with active fractions, but both the precipitates and the filtrates regenerated from mercuric sulfate precipitation have always proved inactive. It was necessary, therefore, to devise other methods for concentrating this fraction.

Although the leucine-like fraction was least soluble in the cold in the presence of small amounts of alcohol, the fraction precipitable by mercuric sulfate is least soluble in the presence of higher concentrations of alcohol and ether at a higher acidity and temperature. The precipitate resulting when 11 volumes of alcohol and 6 of ether were added to 1 volume of water consisted largely of such material, and, when sufficiently extracted with this water-alcohol-ether mixture, no longer contained any appreciable amount

of α-amino nitrogen, as judged by Van Slyke's method. It also showed no increase in free carboxyl groups upon the addition of formaldehyde. 600 mg. of this fraction have been intravenously injected and have proved extremely active.

Presumably, therefore, the active principle effective in pernicious anemia is not an amino acid but a nitrogenous base, the nitrogen in which exists as in a secondary or tertiary amine. Since the nitrogen content of the purest fractions have been in the neighborhood of 10.8 per cent, purine or pyrimidine bases appear to be excluded but not ring compounds of the pyrrole or pyridine types. Crystallization and identification of the active principle remains to be accomplished.

PLASMA PHOSPHATASE IN DISEASES OF BONE.

By H. D. KAY.

(From the Department of Biochemistry, University of Toronto, Toronto, Canada.)

In cases of bone disease involving a large part of the skeleton there is usually a marked rise above the normal in the amount of the plasma phosphatase. In every one of twenty-four cases of osteitis deformans the quantity of this enzyme was increased; in 50 per cent of the cases it was increased to more than 10 times the normal. The rise is almost absolutely specific for diseases involving the skeleton.

This is of considerable interest in connection with the theory that the phosphatase present in bone is one of the essential factors in the processes of bone formation and maintenance. Several cases of "renal" and "adolescent" rickets show the same marked rise as does one certainly established case of hyperparathyroidism. In exophthalmic goiter and hyperthyroidism, though the rise is not to the osteitis level, there is frequently a definitely increased plasma phosphatase.

THE ISOERGOSTEROLS AND VITAMIN D.

By CHARLES E. BILLS, FRANCIS G. McDONALD, and WARREN M. COX, Jr.

(*From the Research Laboratory, Mead Johnson and Company, Evansville, Indiana.*)

Ergosterol in benzene solution is isomerized by dehydrated fullers' earth. The product differs in specific rotation from the isoergosterols obtained with acid catalysts. All these isomers, formerly regarded as distinct, intertransformable substances, appear actually to be mixtures of at least two forms—the mutual transformations being the establishment of equilibria between the forms. A by-product of the action of fullers' earth is ergosteryl isoergosteryl ether. This ether exhibits ergosterol absorption bands, yet it cannot be activated, and so we have evidence of the importance of the hydroxyl group in activation. The earliest detectable photodecomposition product of vitamin D is a substance exhibiting an absorption band and an extinction coefficient which apparently identify it with isoergosterol. Vitamin D may therefore be regarded as isomeric with ergosterol.

METABOLIC RATES AND RESPIRATORY QUOTIENTS OF RATS FOLLOWING THE INGESTION OF DEXTRIN.*

By LAURENCE G. WESSON.

(*From the Department of Pharmacology, Vanderbilt University School of Medicine, Nashville.*)

The metabolic rates and respiratory quotients of twenty-five rats were obtained for 44 hours following the ingestion of sufficient dextrin to furnish the rats with the required calories for 12 hours. All determinations were made at 28° in the absence of light after the rat had remained for 3 or 4 days under these conditions. The glycogen stores of the rats were depleted by 18 to 24 hours fasting before they were fed dextrin. Metabolic rate and respiratory quotient determinations were made both with the Henderson-Haldane gas analysis apparatus and an improved form of the closed circuit calorimeter previously described by us. The widely varying values of individual rats both as regards metabolic rates and respiratory quotients in the first hours after the test meal

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became gradually more uniform, and from the 27th and 36th hour the deviations were comparatively small for respiratory quotients and metabolic rates respectively. An average, uncorrected R.Q. value of 0.73 to 0.74 was obtained regularly following the 37th hour. The final metabolic rate value was approximately 720 calories per sq. m. of body surface per 24 hours.

PLASMA pH IN CANCER.*

By FRITZ BISCHOFF, M. LOUISA LONG, AND ELSIE HILL.

(From the Chemical Laboratory of the Potter Metabolic Clinic, and the Department of Cancer Research, Santa Barbara Cottage Hospital, Santa Barbara.)

The plasma pH of fifteen normal individuals, fifteen cancer patients who had received no treatment, and ten cancer patients who had taken radium, x-ray, or lead treatment, was determined directly by means of the quinhydrone electrode at room temperature. The results were corrected to the oral temperature of the subject for comparison. The normal group showed an average plasma pH of 7.46 with a minimum value of 7.43 and a maximum value of 7.50. The cancer group, which had received no treatment, showed an average pH of 7.50, with a minimum value of 7.44 and a maximum value of 7.59. Two-thirds of the values of this group were above the highest value obtained in the normal group. The cancer group under treatment showed an average pH of 7.52. The findings do not agree with Millet who reported no difference between his normal and cancer group.

BLOOD CHEMISTRY STUDIES WITH HENS BEARING ROUS SARCOMA NO. 1.*

By JOSEPH H. ROE.

(From the Department of Biochemistry, George Washington University Medical School, Washington.)

Chemical analysis of the bloods of hens bearing Rous sarcoma No. 1 showed no variations from control values in non-protein nitrogen, uric acid, creatinine, chlorides, cholesterol, serum calcium, and inorganic phosphorus, hemoglobin and bilirubin, the

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analyses being performed at times varying from 18 days before death to the day of death. A distinct elevation of the blood sugar level was noted. Studies of the enzymes concerned in carbohydrate metabolism showed no variation from control values for the glycogenolytic enzymes of the blood of the sarcoma-bearing hens, but a very marked increase in the glycolytic activity of the bloods of the tumor-bearing fowls.

ALKALOSIS OBSERVED IN CASES WITH PERSISTENT HYPERTENSION.*

By EDWARD MUNTWYLER and CHARLES T. WAY.

(From the Department of Biochemistry, School of Medicine, Western Reserve University, Cleveland.)

An acid-base equilibrium study of the plasma has been made on cases with persistent hypertension and with only slight nitrogen retention. A report of three cases is made, and at the present time a study is being conducted on two more cases showing similar results. If one assumes 31.0 mM as the upper limit for a normal bicarbonate concentration there was in each case a condition of alkalosis at some period in the observation. The first case showed an uncompensated alkalosis over a period of 22 days just preceding death, with the pH varying between 7.54 to 7.56 and the bicarbonate from 34.2 to 36.6 mM. The second case though not presenting as severe an alkalosis as the first gave 34.5 mM as the highest observed bicarbonate concentration. Both cases were characterized by a progressive fall of chloride and total base. There was a greater decrease of chloride than of total base. Since both of these cases showed periodic vomiting, the latter may have been the paramount factor in producing the bicarbonate excess, though this cannot be accepted as final as no analyses of the vomitus were made. The third case differed from the first two in that there was no progressive decrease of the chloride and total base concentrations, and further, vomiting was not present. In spite of a normal concentration of chloride there was a bicarbonate concentration of 28.3 to 32.2 mM existing over a period of 4 months. From that time on there was a progressive decrease in bicarbonate to give a terminal uncompensated acidosis. Since the total base

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in this case remained normal the undetermined acid concentration (phosphates, sulfates, and organic acids) showed a progressive increase.

CALCIUM AND PHOSPHORUS METABOLISM IN RELATION TO CERTAIN BONE DISEASES.

II. HYPOCALCURIA.

By GENEVIEVE STEARNS and JULIAN D. BOYD.

(From the Department of Pediatrics, College of Medicine, State University of Iowa, Iowa City.)

Patients with late rickets have been observed to exhibit a hypocalcuria when given neutral diets, the urinary calcium being practically unchanged whether the intake of calcium be low or high. This apparent decrease in ability to excrete calcium in the urine has been noted also in two patients with arthritis and in one patient with hypothyroid, hypoparathyroidism. One patient with myxedema exhibited a very low urinary calcium when the intake was low, but on increasing the calcium of the diet, the urinary calcium increased to nearly the normal level.

THE INTERMEDIARY METABOLISM OF PHENYLALANINE.

By JOSEPH P. CHANDLER and HOWARD B. LEWIS.

(From the Laboratory of Physiological Chemistry, Medical School, University of Michigan, Ann Arbor.)

From the urine of rabbits fed 2 gm. of *l*- or *dl*-phenylalanine daily, phenylpyruvic and phenaceturic acids have been isolated and the amounts present determined quantitatively. No increase in benzoic acid (present as hippuric acid) in the urine following oral administration of phenylalanine was observed. The increased amount of phenylacetic acid (present as phenaceturic acid) found after the administration of phenylalanine would indicate that the benzene nucleus of phenylalanine is not as readily or as completely oxidized as is usually assumed.

CYSTINURIA AND TUBERCULOSIS.

By HOWARD B. LEWIS and MARIE F. O'CONNOR.

(*From the Laboratory of Physiological Chemistry, Medical School, and the Department of Laboratories, University Hospital, University of Michigan, Ann Arbor.*)

It has been stated[27] that cystinuria is associated in many cases with tuberculosis, cystine having been isolated from more than 40 per cent of the urines of the tuberculous subjects examined. We have examined a considerable number of urines from tuberculous subjects by the Sullivan reaction for cystine applied as a qualitative test. In no case have we obtained a reaction in the urine differing from the negative reaction of normal urines. The examinations were controlled by simultaneous tests on normal urines to which cystine has been added in small amounts, which gave uniformly positive results. Quantitative examinations of many of these same urines by a modification of the Folin-Marenzi method showed in no case an amount of cystine beyond the range of the variations found in normal subjects.

THE COLLOIDAL OSMOTIC PRESSURE OF BLOOD IN DIABETES MELLITUS.

By I. M. RABINOWITCH.

(*From the Department of Metabolism, Montreal General Hospital, Montreal, Canada.*)

In diabetic blood, the colloidal osmotic pressure per gm. of protein may be increased. This is attributed to lipemia. In the absence of lipemia, as measured by cholesterol, the colloidal osmotic pressure per gm. of protein was found to be normal. In the absence of marked edema, the protein concentration of diabetic blood is normal; with lipemia, therefore, in the absence of edema, the total colloidal osmotic pressure of the blood is increased. If the prevalent view of urinary excretion is correct, in the diabetic with lipemia, a greater hydrostatic pressure must be necessary to overcome the colloidal osmotic pressure in the capillaries before glomerular filtration can occur, that is, before urine can be excreted. It is suggested that this increased pressure, though rela-

[27] Monceaux, R., *Compt. rend. Soc. biol.*, **96**, 323 (1927).

tively small, when, however, continued over a long period of time, probably has the same effect as more marked intracapillary pressure exerted over a short period of time. The latter, when produced either by injection of adrenalin or sympathetic stimulation, has been alleged to produce arteriosclerosis. Is this, therefore, a cause of the marked incidence of cardiovascular disease in diabetes? Clinical and experimental data are presented which tend to favor this view.

THE MECHANISM OF THE RECOVERY PROCESS AFTER EXERCISE IN MAMMALS.

By C. N. H. LONG and RHODA GRANT.

(From the University Clinic, Department of Medicine, McGill University, Royal Victoria Hospital, Montreal, Canada.)

We have been studying the changes in liver and body glycogen and lactic acid in rats after exercise, using the methods introduced by Cori and Cori.[28] The rats were all fasted for 24 hours beforehand and were exercised artificially in such a manner that conditions from animal to animal were comparable. The exercise was sufficient to set up large oxygen debts. No anesthetic was used. To date the results are as follows: (1) Body glycogen decreases as a result of exercise and is only slowly restored. 5 hours after exercise the glycogen content is still less than before exercise but 24 hours later (food withheld) it is again within normal limits. (2) Liver glycogen either decreases or remains unchanged after exercise that has reduced body glycogen by two-thirds its initial value. During recovery there may be a slight increase in liver glycogen but this is not very marked. (3) The lactic acid formed as a result of exercise is removed long before the glycogen content of the body has returned to normal. Thus 2 hours after exercise the glycogen stores of the body were 50 per cent less than their initial value while the lactic acid content was normal. (4) A second period of exercise at a time when the body stores of glycogen were low (1 to 2 hours after the first period of exercise) resulted in the appearance of lactic acid in amounts nearly equal to those produced by the first period of exercise when the body stores of glycogen were relatively high. (5) The intravenous injection of *d*-lactic acid

[28] Cori, C. F., and Cori, G. T., *J. Biol. Chem.*, **76**, 755 (1928).